MAGYAR BOTANIKAI LAPOK.

(UNGARISCHE BOTANISCHE BLÄTTER.)

KIADJA ÉS SZERKESZTI: — HERAUSGEBER U. REDAKTEUR:

Dr. DEGEN ÁRPÁD.

FŐMUNKATÁRSAK: — HAUPTMITARBEITER:

Dr. GYŐRFFY ISTVÁN — Dr. LENGYEL GÉZA.

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3 TÁBLÁVAL S 1 SZÖVEGKÖZÖTTI RAJZZAL — MIT DREI TAFELN UND 1 TEXTILLUSTRATION.

ROYAL BOTANIC GARDENS
S.O.
C.20.10.23.
KEW

BUDAPEST, 1923.

Mykologiai közlemények. V.[1])

Mykologische Mitteilungen. V.

(3 táblával — mit 3 Tafel)

Irta: } G. von Moesz.
Von: }

48. Pilze aus der Sammlung von Á. v. Degen.

Schon längere Zeit habe ich die Gelegenheit mich mit jenen Pilzen zu befassen, die von Árpád v. Degen an verschiedenen Orten gesammelt wurden. Zum grösseren Teil stammen dieselben aus dem Velebit-Gebirge. Da er aber die Flora dieses Gebietes in einem selbstständigen Werke zu veröffentlichen gedenkt, vermeide ich an dieser Stelle eine Aufzählung der aus diesem Gebiete stammenden Arten. Ich bemerke noch, dass diese Mitteilung nur einen Teil der von Á. v. Degen gesammelten Pilze umfasst. Dieselben liegen im Herbar des Ung. Nationalmuseums auf.

a) Pilze aus Ungarn.

Schizomycetes.

Pseudomonas mucilaginosus-Koeleriae (Aujeszky) Moesz in Bot. Közl. (1915) XIV. 158. — Szigetszentmiklós, Insula Csepel fl. Danubii, in spicis vivis *Koeleriae glaucae* var. *dactyloidis*.

[1]) I—IV. Közlem. megjelent a Bot. Közl. XII., XIV., XVII. és XIX. évfolyamaiban.
Die Mitteilungen I—IV. erschienen in der Jahrgänge XII., XIV., XVII. und XIX. der „Botanikai Közlemények".

Ascomycetes.

Xyloma salicinum Pers. — Hohe Tátra, Bélaer Alpen: Stierberg. In foliis *Salicis Jacquinianae.* (Status conidiiferus.) Da v. Hőhnel bewiesen hat (Ann. Mycol. XV. 1917. 316), dass jene Arten die bisher zum Genus *Rhytisma* gezählt wurden, in sechs sich von einander scharf unterscheidende Genera gehören, muss auch *Rhytisma salicinum* an seine ursprüngliche Stelle in das Genus *Xyloma* Persoon zurück versetzt werden.

Scleroderris aggregata (Lasch) Rehm. — Pomáz: Dobra Voda. In caulibus vivis *Euphrasiae tataricae.* — Asci noch unentwickelt. Konidien farblos, zweizellig, zylindrisch, 13—29×3—3·5 μ gross, Diplodina-artig. Diese Konidien scheinen identisch mit jenen zu sein, welche von Fuckel beschrieben und zu *Cenangium aggregatum* gehörig befunden worden sind. (Fuckel: Symb. Myc. 271). In Ungarn wurde die Art bisher nur von Hazslinszky bei Bártfa an Stengeln von Euphrasia odontites gefunden. Rehm bezweifelt zwar (in Rabh. Kryptog. fl. I. III. 212. No. 4677) die Richtigkeit der Hazslinszky'schen Angabe — und nach Hazslinszky's Beschreibung (Verh. Zool. Bot. Ges. Wien, 1887, XXXVII. 159) konnte er auch zu keiner anderen Folgerung gelangen — doch auf Grund der Untersuchung des im Ung. Nat. Museum befindlichen Originalexemplars halte ich den z aus Bártfa ohne Zweifel für *Scleroderris aggregata.* Unverständlich ist nur, dass Hazslinszky die farblosen Sporen als braun angibt.

Lophodermium alpinum Rehm — Bucsecs. In foliis *Festucae saxatilis,* leg. Ginzberger. — Die kleinen elliptischen Apothezien und den kurzen Sporen (40—43 μ) zufolge halte ich den Pilz für *L. alpinum,* obwohl die Schmalheit der Sporen (1·5—2 μ) auch L. culmigenum nicht ausschliesst.

Erysibe cichoriacearum DC. — Kispest. In foliis *Hyoscyami nigri.* Status conidiiferus.

Erysibe polygoni (DC) Schröter — Prope Pomáz. In foliis *Trifolii alpestris.*

Claviceps nigricans Tul. — Szabadszállás. In ovariis *Heleocharidis palustris.* Sclerotium.

Epichloë typhina (Pers.) Tul. — Horgos, (Comit. Csongrád). In vaginis *Atropidis limosae.*

? *Arcangelia hepaticarum* Sacc. — Pomáz, zusammen mit *Riccia natans* im Wasser schwebend, in grosser Menge. Im Innern der schwarzen, rundlichen Fruchtkörper waren Fortpflanzungsorgane noch nicht entwickelt. Darum kann ich die Bestimmung nur mit Vorbehalt mitteilen. Diese Fundstelle sollte noch beobachtet werden, um zu einem sicheren Ergebnis zu gelangen. Der Pilz wurde bisher nur in Italien gefunden.

Mycosphaerella violae A. Potebnia in Ann. Myc. (1910) VIII. 51. — In monte „Öcsém teteje" in foliis emortuis *Violae*

alpinae, 18. Jun. 1901. — Peritheciis 72—116 μ diam., ascis 62—65×20—23 μ, sporis 20—23×3—5 μ. — Ein erhöhtes Interesse verleiht dem Pilze dieses Fundortes jener Umstand, dass die Art bisher nur in der Kultur gezogen wurde. Dass die Art auch in der Natur vorkommt, wird durch den von Degen gefundenen Pilz bewiesen.

Omphalospora melaena (Fries) v. H.: Fr. z. Myk. (1919) No. 1179. — *Ascospora melaena* Fries — Monor. In foliis caulibusque *Astragali Onobrychidis*.

Pyrenophora dianthi (De Not.) Berl. Székelykő. In foliis, caulibusque *Dianthi spiculifolii*.

Basidiomycetes

Ustilagineae.

Cintractia caricis (Pers.) Magnus. — Solymár. In ovariis *Caricis Michelii*. In monte „Kis Szénáshegy" prope pag. Pilisszentiván. In ovariis *Caricis flaccae*.

Cintractia subinclusa (Koern.) Magn. Insula Csepel fluv. Danubii, prope opp. Soroksár. In ovariis *Caricis ripariae*.

Sphacelotheca andropogonis (Opiz) Bubák. — In monte „Kis Csikóvár" prope pag. Pomáz. In inflorescentia *Andropogonis Ischaemi*.

Urocystis anemones (Pers.) Winter — Törökbálint. In foliis *Hellebori dumetorum*.

Ustilago holostei De By. — Supra pagum Szvinica. In ovariis *Holostei umbellati*.

Ustilago major Schroeter — Halomegyháza puszta, inter pag. Üllő et Gyál. In antheris ovariisque *Silenes parviflorae*.

Uredinales.

Uromyces ambiguus (DC) Lév. — Insula Csepel, fluv. Danubii. In foliis *Allii Scorodoprasi*.

U. astragali (Opiz) Sacc. — Tököl, insulae Csepel. In foliis *Oxytropidis pilosae* var. *hungaricae* Borb. In monte Naszál, prope opp. Vácz, in foliis *Astragali glycyphylli*.

U. ficariae (Schum.) Lév. — Hűvösvölgy, prope Budapest. In foliis *Ranunculi Ficariae*.

U. fulgens (Hazsl.) Bub. — In monte Rókahegy, prope pag. Békásmegyer. In ramis *Cytisi austriaci*.

U. minor Schroet. — In monte Kis Csikóvár, prope pag. Pomáz. In foliis *Trifolii montani*.

Puccinia asparagi DC. — In monte Pismányhegy, prope opp. Szentendre. In caulibus *Asparagi officinalis*.

P. bistortae (Str.) DC. — In monte Nagy-Hagymás, comit. Csik. In foliis *Polygoni vivipari*.

P. drabae Rud. — „Egyeskő" prope pag. Pilisszentiván. In caulibus, petiolisque *Drabae lasiocarpae* [Edit. in Fl. Hung. exs. Cent. V. (1919) No. 404].

P. grisea (Str.) Winter — „Farkasvölgy", prope Budapest. In foliis *Globulariae Willkommii.*

P. lolii Niels. — Békásmegyer. Aecidium in foliis *Rhamni catharticae.*

P. menthae Pers. — In monte Naszál, prope opp. Vácz. In foliis *Calaminthae Acini.*

P. obscura Schroeter. — In monte Kis Csikóvár, prope pag. Pomáz. In foliis *Luzulae albidae.*

P. phragmitis (Schum.) Koern. — Insula fluv. Danubii: „Molnársziget" prope opp. Soroksár. In foliis *Phragmitis communis.*

P. taraxaci (Rebent) Plowr. — Békásmegyer. In foliis *Taraxaci serotini.*

P. thesii (Desv.) Chaill. — In monte Kőhegy prope pag. Pomáz. Aecidium in foliis *Thesii intermedii.* — In monte Harsányhegy, prope pag. Villány. Aecidium in fol. *Thesii sp.* Eventuell kann der Pilz auch *P. Passerini* Schroet. sein.

P. violae (Schum.) DC — „Nagy Nyir" prope opp. Kecskemét. Aecidium in foliis *Violae cyaneae.*

Coleosporium euphrasiae (Schum.) Winter — Insula „Molnár sziget" fluvii Danubii, prope opp. Soroksár. In foliis *Alectorolophi goniotrichi* Borb. — Halomegyháza puszta, in fol. caulibusque *Alectorolophi montani.*

Melampsora lini (Ehrenb.) Lév. — In monte Kis Szénáshegy, prope pag. Pilisszentiván. In foliis *Lini cathartici.*

Phragmidium fragariastri (DC) Schroeter. — Detrekő Csütörtök (Com. Pozsony). In foliis *Potentillae albae.*

Polyporaceae.

Polystictus perennis (L.) Fries. — „Nagy erdő" prope opp. Nagykőrös.

Gasteromycetes.

Geaster saccatus Fries. — In arenosis prope Monor.

Tylostoma granulosum Lév. — In arenosis silvae prope Monor.

T. mammosum (Mich) Fr. — Monor.

Fungi imperfecti.

Sphaeropsidales.

Cylindrosporella alnea (Lév.) v. H. — *Leptothyrium alneum* Sacc. — Dömörkapu, prope opp. Szentendre. In foliis vivis *Alni glutinosae.*

Dilophospora graminis Desm. Insula „Csepelsziget." In vaginis et inflorescentia *Koeleriae glaucae* var. *dactyloidis.*

Diplodina gypsophilae Hollós. „Nagy Nyír" prope opp. Kecskemét. In caulibus siccis *Dianthi serotini.* Hollós fand diesen Pilz ebendaselbst, doch auf Stengeln von *Gypsophila paniculata.*

Septoria asaricola Allescher — „Hűvösvölgy“ prope Budapest. In foliis *Asari europaei.*

Melanconiales.

Pestalozzia funerea Desm. — Budapest. In foliis *Pini Pumilionis.* Der Pilz richtete in A. v. Degens Garten die *P. Pumilio* Sträucher zugrunde.

Hyphomycetes.

Bostrychonema alpestre Ces. — In monte Nagy-Hagymás, Comit. Csik. In foliis *Polygoni vivipari.*

Botrytis Bassiana Bals. — In monte „Naszál“ prope opp. Vácz. In corpore emortuo *Coccinellae septempunctatae.*

Epicoccum purpurascens Ehbg. — Dunaharaszti. In foliis emortuis *Typhae latifoliae.*

Fumago vagans Pers. — In monte Pismányhegy, prope Szentendre. In fructibus *Asparagi officinalis.*

Helminthosporium gracile Sacc. — Erény. In foliis *Iridis spuriae.*

Oidium erysiphodes Fr. — Izbég. In foliis caulibusque *Lithospermi arvensis.*

Oidium quercinum Thümen — In monte „Kis Csikóvár“ prope Pomáz. In foliis *Quercus lanuginosae.* — „Nagy Nyír“, prope opp. Kecskemét. In fol. *Quercus Roburis.*

Ramularia macrospora Fres. — Deliblat. In foliis *Campanulae persicifoliae.*

Tubercularia confluens Pers. — Budapest. In cortice *Aesculi Hippocastani.*

Mycelium sterile.

Sclerotium stercorarium DC. — Budapest (Kőbánya). In dejectionibus humanis.

Sclerotium sp. — Budapest. In petiolibus foliorum *Robiniae Pseudacaciae.*

b) **Aus anderen Ländern von Europa.**

Uredinales.

Puccinia bullata (Pers.) Winter — Ragusa, Dalmatiae. In foliis *Seseleos tomentosi* Vis.

P. herniariae Unger — Vranja, Serbiae. In foliis vivis *Herniariae hirsutae.* Legit Adamović.

P. menthae Pers. — In monte Pindus, Graeciae. In foliis *Satureiae parnassicae.* Legit R. Maire.

P. Mougeotii Lagh. — In monte Plješivica, prope Korenica, Croatiae, uredo et teleutosporae in foliis *Thesii alpini.*

Uromyces salsolae Reichardt. — Rossia. Gub. Tambow. In foliis *Salsolae Kali.* Legit Schirajevsky (Ex Herb. Fl. Ross. XVII. No. 837).

Sphaeropsidales.

Darluca filum (Biv.) Cast. — Rossia. Gubern. Tambow. In soris *Uromycis salsolae*. Leg. Schirajevsky.

Hendersonia tragacanthae Delacr. — Insula Samothrake, in monte Phengari. In spinis *Astragali Parnassi* Boiss.

Phyllosticta hederacea (Arc.) Allescher — Abbazia, Istriae. In foliis *Hederae Helicis*.

Septoria Rajkoffi Bubák et Moesz.

Syn: *Septoria Rajkoffii* Bubák nomen nudum in Sacc. Syll. Fung. XXII. (1913) p. 1112. Exsicc.: Kabát et Bubák: Fungi imperf. fasc. XI. (1909) No 522.

Maculis amphigenis, suborbicularibus vel irregulariter oblongis, ochraceo-alutaceis, arescentibus, brunneo vel purpureo marginatis; pycnidiis plerumque epiphyllis, gregariis, immerso-prominulis, globulosis, 65—115 μ diam., tenui membranaceis, contextu parenchymatico, flavido-brunneo; conidiis filiformibus, apice angustatis, plerumque leniter flexuosis, hyalinis, continuis vel 1—3-septatis, 19—40 × 1·5 — 2·5 μ; conidiophoris papilliformibus, 7×2—3 μ.

Hab. in foliis *Cynanchi acuti* ad Sadovo Bulgariae (leg. Dr. F. Bubák, 13. IX. 1907) et ad Constanza Romaniae (leg. J. Prodan, 22, VII. 1910).

Aus Zuvorkommenheit des Herrn Dr. F. Bubák, hatte ich die Gelegenheit den Pilz aus Sadovo mit jenem aus Constanza vergleichen zu können. Ich fand, das beide Pilze identisch sind. Es war der Wunsch des Herrn Prof. F. Bubák, das ich die Diagnose verfasse und das ich die Mitautorschaft übernehme.

(Abbildung: Tab. I)

Erklärung zu Abbildung 1: *Septoria Rajkoffi Bubák*; *A* Blatt in nat. Grösse; *B* Querschnitt, mit zwei Pycnidien, 100-mal vergr.; *C* Konidien, 700-mal vergr.

Septoria ribis Desm. — In valle Rosegg, prope Pontresinam, Helvetiae. In foliis *Ribis petraei*.

Melanconiales.

Gloeosporium nobile Sacc. — Abbazia, Istriae. In foliis *Lauri nobilis*. Vereinzelt fand ich in einigen Konidien 1—3 Septa. Würden die Septa allgemein erscheinen, sollte die Art zum Genus *Septogloeum* gezogen werden.

c) **Pilz aus Asien.**

Euryachora tragacanthae (Lév.) Theiss. et Syd. — In valle Lur, ad pagum Getschesär montium Elburs, Persiae borealis. In spinis *Astragali talagonici*. Legit Bornmüller.

49. Phaeochora sphaerotheca (Earle) Moesz.

Synon: *Anthostomella sphaerotheca* Earle in Bull. Torr. Bot. Cl. (1898) p. 360.
Phyllosticta sabalicola Szabó in Schedae ad Krypt. exs. Vindob. Cent. IX. No. 828 et in Annalen d. Naturhist. Hofmus. XVIII. (1903) p. 356.

(Abbildung: Tab. I)

Prof. S. Mágocsy-Dietz schenkte dem Ungarischen National Museum zahlreiche Exemplare der *Phyllosticta sabalicola* Szabó. Das Material sammelte er im bot. Garten der Universität auf dürren Blattstielen von *Sabal Blackburniana*. Das schöne Material verlockte mich zur näherer Untersuchung des Pilzes, welchen ich übrigens mit der, im „Krypt. exicc. Vindob." unter No. 828 herausgegebenen Exemplare vollkommen identisch fand.

Der Fruchtkörper ist stromatisch. Von Pycnidien ist keine Spur vorhandan. Das Stroma ist in kleinerem Teil im Epidermis in grösserem Teil im Gewebe unter dem Epidermis ausgebreitet. Im Querschnitt zeigt der Fruchtkörper einen Loculus, im Längsschnitt mehrere (2—3). Die Cuticula, respektive die Epidermis deckt das Stroma fest und ständig, nur in der Mitte springt es mehr-minder mit rundlicher Öffnung auf. In dem Loculus sind die einzelligen blassbraunen, 12—18×3—5 μ grossen elliptischen, oder spindelförmigen Sporen in grosser Zahl zu sehen, welche eine grosse, zentralgelegene Guttula enthalten. Zwischen diesen findet man frei einen oder einige rundliche, sehr dünnwandige Asci, deren Grösse 20—26×13—18 μ misst. Die Asci enthalten 8 Sporen, welche gedrängt, gruppenweise im Ascus Platz nehmen, dessen Raum vollkommen ausfüllend. Die Wände der Asci gehen schnell zu Grunde, und es werden dann die Sporen frei.

Auf Grund der hier beschriebenen und nach der, in der Abbildung 2 dargestellten Construktion muss dieser Pilz in die Familie der *Dothideales* — *Phyllachoraceae* gestellt werden, und zwar in die Gattung *Phaeochora* v. H. Die bisher bekannten Arten dieser Gattung aber unterscheiden sich scharf von diesem Pilz. Da nun aber bekannte Tatsache ist, dass die Gattung *Phaeochora* zu den Gattungen *Auerswaldia* und *Anthostomella* nähere Beziehungen unterhält, suchte ich auch unter ihnen nach einer, dem *Phyllosticta sabalicola* ähnlicher Form. Ich fand auch diesen Pilz in *Anthostomella sphaerotheca* Earle, welcher in Nord-Amerika auf abgestorbenen Blattstiele von *Sabal Adansoni* gefunden wurde. Auf Grund der Beschreibung dieses Pilzes zweifle ich nicht, dass diese *Anthostomella* auch eigentlich eine *Phaeochora* ist, umsomehr, da Earle auch selbst sagt, dass dieser Pilz, wegen seinem gut ausgebildeten Stroma und den unvollkommenen Perithecien an die Dothideaceae erinnert. Die Übereinstimmung der runden und schnell vergänglichen Asci, ihrer Grösse und die Dimensionen der Sporen, sowie die übrigen Charaktere beweisen ohne Zweifel,

dass *Phyllosticta sabalicola* Szabó mit *Anthostomella sphaerotheca* Earle identisch ist, welcher Pilz aber in die Gattung *Phaeochora* versetzt werden muss.

Erklärung zu Abbildung 2: *A* Ein Teil eines Blattstieles von *Sabal Blackburniana*, nat. Grösse; *B* Fruchtkörper von oben gesehen, 9-mal vergr.; *C* Querschnitt durch einem Fruchtkörper, *D* Längsschnitt; beide 150-mal vergr.; *E* ein Ascus, *F* Sporen. *E* und *F* 1000-mal vergr.

50. Plectophomella visci ? (Sacc.) Moesz.

Synon.: ? *Phyllosticta visci* Sacc.

(Abbildung: Tab. II)

Der Fruchtkörper ist zuerst sklerotiumartig. Der Nucleus lockert sich später in der Mitte und wird lückenhaft. Die Lücken sind unregelmässig geformt und verteilen sich unregelmässig, vermutlich kommen sie durch Verschleimung der Zellen des Nucleus zu Stande. In diese Lücken, welche sich später zu Höhlungen erweitern, reichen die hyalinen, oder gelblichen Gerüstteile des Nucleus hinein, welche teilweise aus runden, verschleimten Zellen, teilweise aber aus fadenförmigen Paraphysen (ascogene Hyphen?) bestehen. Die Höhlungen werden ziemlich dicht ausgefüllt von kleinen, zylindrischen, einzelligen, farblosen Konidien, welche — soweit ich dies beobachten konnte — aus runden Zellen entstehen, jedoch nicht endogen, sondern durch Abschnürung. An den fadenförmigen Gebilden des Nucleus sah ich keine Spur von Konidienbildung, und deshalb kann ich dieselbe auch nicht für Konidienträger halten.

Die Wand des Pycnidiums besteht an der Basis aus dünneren, nach der Spitze aus dickeren und dünkleren, im Durchmesser 4·5—8 μ grossen braunen, eckigen Zellen, die in 3—4 Reihen liegen. Die innere Fläche der Wand besteht aus verblassenden und hyalinischen, winzigen Zellen, welche zur Verschleimung neigen. An diese fügen sich die Konidien erzeugenden runden Zellen, sowie die sterile Fäden an. An die innere Fläche der Wand schmiegt sich stellenweise eine breitere (22—30 μ) hyalinische Schicht an, deren Bau wegen der Verschleimung nicht zu ermitteln war. In Folge der Zerreissung des Nucleus in Lücken scheint es zuweilen, als ob der Fruchtkörper gekammert sein würde. In einigen Fruchtkörpern war die Spur einer Kammerbildung zu beobachten.

Dieser eigentümlicher Bau des Fruchtkörpers führt vermutlich zur Aufstellung einer neuen Gattung. Vorläufig nenne ich diese Gattung *Plectophomella*, deren Existenz-Berechtigung durch das Auffinden und Nachforschen ähnlich gebauter Formen, entschieden werden wird.

Das Fehlen der Konidienträger und die Verschleimung der Zellen des Nucleus, würde diese Gattung in die Familie der *Sclerophomaceae* hinweisen, wenn die Konidien auf endogener Weise entstehen würden. Auch frägt es sich, ob man den Fruchtkörper dieses Pilzes für stromatisch halten kann? Die ausgebildeten Fäden erinnern an *Plectophoma* und ihrer Verwandtschaft *(Dendrophoma, Pleurophoma)*, doch sind deren Fäden Konidienträger. Paraphysen erwähnt, soweit es mir bekannt ist, nur Spegazzini in den Pycnidien von *Sphaeronaemopsis*. Diese Gattung aber weicht in allen anderen Beziehungen von *Plectophomella* wesentlich ab.

Jenen Pilz, welcher in dem von Kabát und Bubák herausgegebenen Exsiccatenwerk „Fungi imperfecti exsiccati" unter No 805, als *Phyllosticta visci* Sacc. erschienen ist, halte ich ebenfalls für *Plectophomella*. Das Originalexemplar Saccerdo's hatte ich nicht Gelegenheit zu untersuchen, aber es ist anzunehmen, dass auch dieses hieher gehört.

Plectophomella Moesz *nov. gen.* **Sphaerioidearum.** *Pycnidia erumpentia, ostiolata, membranacea, nucleum e cellulis globoideis mucescentibus atque paraphysibus filiformibus constantem continentia; conidia hyalina, continua, minuta, cylindracea, ut videtur e cellulis globoideis oriunda; conidiophora nulla.*

Plectophomella visci Moesz — *Syn.:?* **Phyllosticta visci** Sacc. — *Maculis amphigenis, orbicularibus, 5—8 mm. latis, albescentibus, brunneo marginatis; pycnidiis amphigenis, gregariis epidermide tectis, demum ostiolo erumpente, globosis, subdepressis, nigris, 116—350 μ diam.; contextu parenchymatico, fumoso-fusco; conidiis numerosis, minutis, 4—7·5×1·5—2 μ, hyalinis, cylindraceis, apice rotundatis, continuis, rectis vel leniter curvulis, eguttulatis vel minute biguttulatis et ut videtur e cellulis nuclei globoideis mucescentibus oriundis; conidiophoris nullis; paraphysibus filiformibus, continuis vel septatis, simplicibus vel parce ramosis, hyalinis, 15—38 μ longis, 3·5—4·5 μ crassis.*

Hab. in foliis vivis et siccis **Visci albi**. *Tata-Tóváros, Hungariae. (Legit* Moesz).

Erklärung zu Abbildung 3: *A* Habitusbild in nat. Grösse; *B* Querschnitt durch eine Reihe von Fruchtkörper, 50-mal vergr.; *C* Durchschnitt durch den Fruchtkörper, 100-mal vergr.; *D* Die äussere und innere Fläche der Fruchtkörperwand, 800-mal vergr.; *E* Ein Partikel des Gerüstes des Nucleus, 1000-mal vergr.; *F* Konidien 1000-mal vergr.

51. Pyrenopeziza lini Moesz.

(Abbildung: Tab. II)

Ascomatibus gregariis, sessilibus, primo innatis, clausis, globosis, dein erumpentibus, cyathiformibus, siccis margine

involutis, humidis apertis, extus nigris, margine albido, elevato crenutato, 230—300 μ latis, disco pallide fusco, contextu parenchymatico; ascis oblongo-clavatis, breve stipitatis, 8-sporis, 40—55×8—10 μ, apice jodo non coerulescentibus, paraphysibus filiformibus, 3 μ crassis, apice non incrassatis; sporidiis distichis, ellipsoideis, utrinque attenuatis, continuis, hyalinis, minute 3—4 guttulatis, 11—14×3 μ.

Hab. in caulibus emortuis Lini austriaci, *prope pag. Szigetszentmiklós, (Insula „Csepel"), Hungariae. (Legit.* Moesz).

Der Pilz lässt sich gut von den bis jetzt bekannten *Pyrenopeziza*-Arten unterscheiden. Auf *Linum* war nur *P. californica* Sacc. [in Ann. Myc. II. (1907) p. 15] bekannt, doch unterscheidet sich die obige Art auch von diesem scharf. Der Rand des Fruchtkörpers ist bei *P. californica* tief gelappt, hingegen bei *P. lini* höchstens schwach gekerbt und spaltet sich nur zufolge Druckes lappig. Das Gehäuse besteht aus parenchymatischen Zellen und nur die äussersten Zellen am Rande sind ein wenig gestreckt, doch sind auch diese mit einander verklebt.

Erklärung zu Abbildung 4: *A* Fruchtkörper von oben gesehen, 50-mal vergr.; *B* Durchschnitt durch den Fruchtkörper, 100-mal vergr.; *C* Verklebte Zellfäden vom Rande des Fruchtkörpers, 500-mal vergr.; *D* Asci und Paraphysen, 500-mal vergr.; *E* Spore, 1000-mal vergr.

52. Sclerophomella harmalae (Hazsl.) Moesz.

Syn.: *Phoma harmalae* Hazsl. (Nomen nudum in herb.)

(Abbildung: Tab. III)

Pycnidiis erumpentibus, subglobosis, in sicco depressis, atris, papillato-ostiolatis, 145—245 μ diam., contextu membranaceo, parenchymatico, fuligineo, extus cellulis angulatis, intus cellulis valde depressis hyalinis; conidiis copiosissimis, cylindraceis, utrinque rotundatis, rectis, biguttulatis, 2—3×1 μ, hyalinis, ex hystolisi mucosa nuclei oriundis.

Hab. in caulibus siccis Pegani harmalae. *Budapest. (Legit* L. Simkovics, *Apr. 1875).*

Erklärung zu Abbildung 4: *A* Durchschnitt durch den Fruchtkörper, 150-mal vergr.; *B* Konidien, 1200-mal vergr.

53. Phomopsis dulcamarae (Sacc.) Trev.

Syn.: *Phoma Scopolinae* Hazsl. (Nomen nudum in herb.)

(Abbildung: Tab. III)

Den in Herbar des Ung. Nat. Museums vorhandenen Pilz sammelte Hazslinszky in Kroatien, bei Lokve auf dürrem Stengel von *Scopolia carniolica.*

Dieses Material ist dadurch interessant, dass es zweierlei Ausbildungen des Stromas vor unsere Augen führt:

1. In der grösseren Anzahl der Fruchtkörper ist das Stroma oben und unten beinahe in gleichmässiger Dicke ausgebildet und oben auch das Peridermium von ihm eingenommen, wodurch der Fruchtkörper in seiner ganzen Grösse sich schwarz gefärbt zeigt. Wenn die Öffnung spaltförmig wäre, könnte man auf ein *Leptothyrium* denken.

2. Bei der kleineren Anzahl der Fruchtkörper ist das Stroma unten dünn oder fehlt es fast; oben ist es dick, ja es tritt hier sogar schnabelartig hervor und breitet sich unter dem Peridermium aus. In diesem Falle ist der Fruchtkörper eine typische *Phomopsis*. Zwischen diesen beiden Formen gibt es auch Übergänge.

Die Konidien der „*Phoma Scopolinae*" von Hazslinszky sind elliptisch, farblos, 6—8×2—3 μ gross, haben zwei Oeltröpfchen. Die Träger der Konidien sind fadenförmig, dünn, 7·5—15 μ lang. Nadelförmige, hackig gekrümmten Konidien sah ich nicht. Auf Grund der Zusammenstimmung der elliptischen Konidien, halte ich den Hazslinszky'schen Pilz identisch mit *Phomopsis dulcamarae* (Sacc.)

Der Umstand, dass die Fruchtkörper der von Sydow in der „Mycotheca Marchica" unter No 4184 herausgegebene „*Phoma Dulcamarae*" fast ausschliesslich nadelförmige Konidien besitzen, spricht nicht gegen diese Identifizierung, da es bekannt ist, dass die zweierlei Konidien der *Phomopsis*-Arten bei einzelnen Fruchtkörpern in verschiedenen Proportionen erscheinen können.

Erklärung zur Abbildung 6: *A* Drei Fruchtkörper im Durchschnitte, 100-mal vergr.; *B* Konidien, mit Träger, 100-mal vergr.

54. Phomopsis malvacearum (West.) Died.

Syn.: *Phoma Hibisci* Hazsl. (Nomen nudum in herb.)

(Abbildung: Tab. III)

Den von mir untersuchten Pilz fand Hazslinszky in Eperjes an abgestorbenen Ästen von *Hibiscus syriacus*. Er stimmt vollkommen mit Diedicke's Beschreibung überein. Ein besonderes Interesse verleiht diesem Exemplar der Umstand, dass die Fruchtkörper neben den spindelförmigen Konidien auch gekrümmte, nadelförmige Konidien besitzen, welche bis jetzt bei dieser Art unbekannt waren. Die Dimensionen dieser Konidien messen: 20—23×1—1·5 μ.

Erklärung zur Abbildung 7: *A* Durchschnitt durch den Fruchtkörper, 100-mal vergr.; *B* Elliptisch-spindelförmige Konidien, sammt Träger, 1000-mal vergr.; *C* Nadelförmige Konidien, 1000-mal vergr.

A 48-ik közleményben a szerző felsorolja azokat a gombákat, amelyeket Degen Árpád különböző helyeken gyűjtött. A Velebit-hegység gombái itt nem szerepelnek, mert azok másutt fognak megjelenni.

A 49-ik közleményben a szerző kimutatja, hogy a „*Phyllosticta sabalicola* Szabó“ nem más, mint *Anthostomella sphaerotheca* Earle, amelyet azonban a *Phaeochora* v. H. génuszba kell áttenni.

Az 50-ik közlemény egy új génusznak, a *Plectophomella*-nak leirását tartalmazza, amelybe a fagyöngy levelének egy gombája tartozik, amely valószinűleg azonos a *Phyllosticta visci* Sacc.-al.

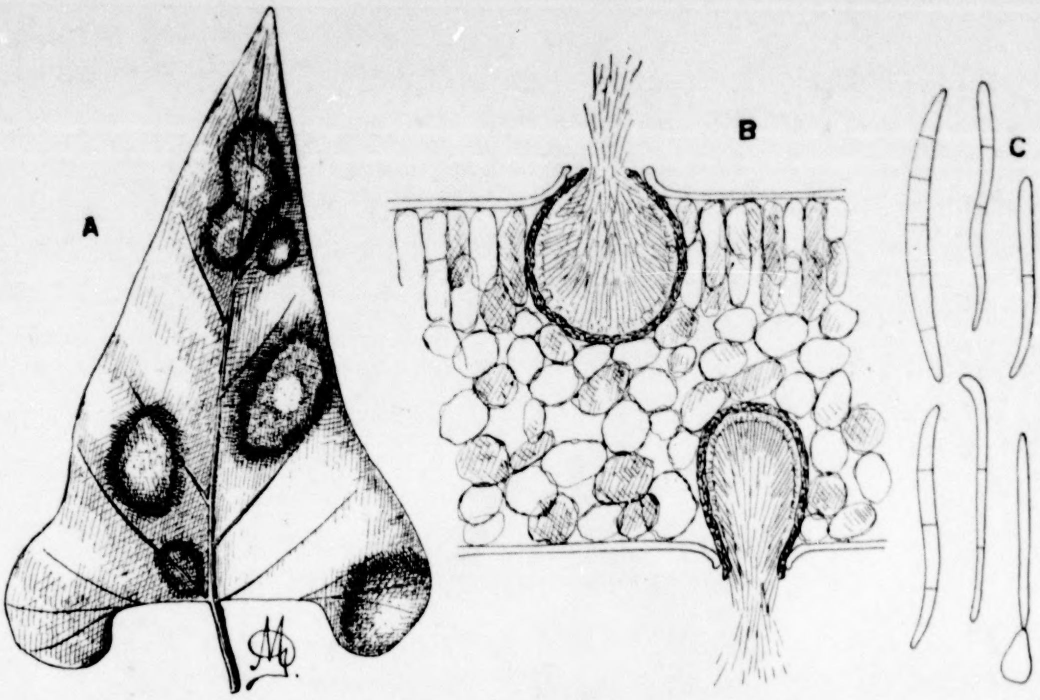
Az 51-ik közlemény a *Linum austriacum* kórójának egyik új gombájának leirását foglalja magában.

Az 52-ik közlemény a Hazslinszky-féle *Phoma harmalae*-nak leirását adja. A szerző ezt a gombát a *Sclerophomella* génuszba helyezi.

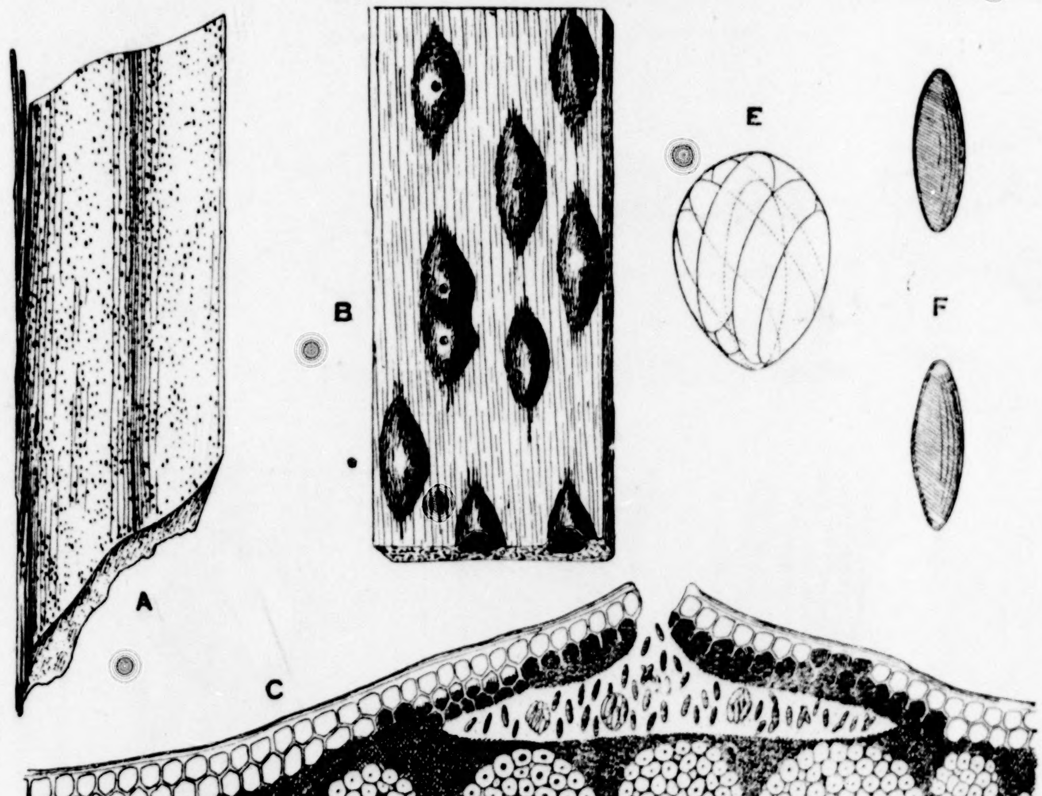
Az 53-ik közleményben a Hazslinszky-féle *Phoma Scopolinae*-t a *Phomopsis dulcamarae*-val azonositja.

Az 54-ik közlemény a Hazslinszky-féle *Phoma Hibisci*-t a *Phomopsis malvacearum*-mal azonositja. A szerző leírja e gomba tűalakú konidiumait, amelyeket eddig nem ismertek.

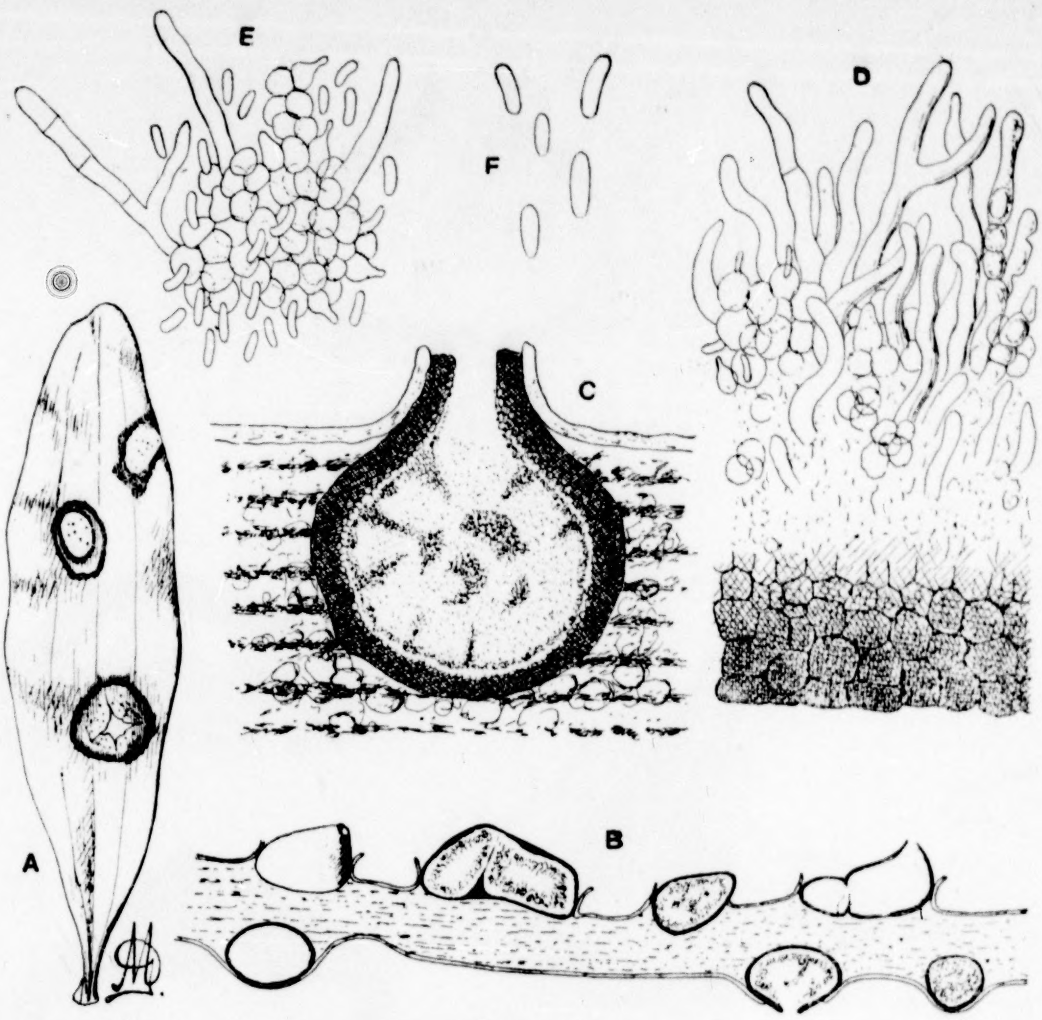
Az ábrák a szövegben leírt új és kritikus gombákat ábrázolják.



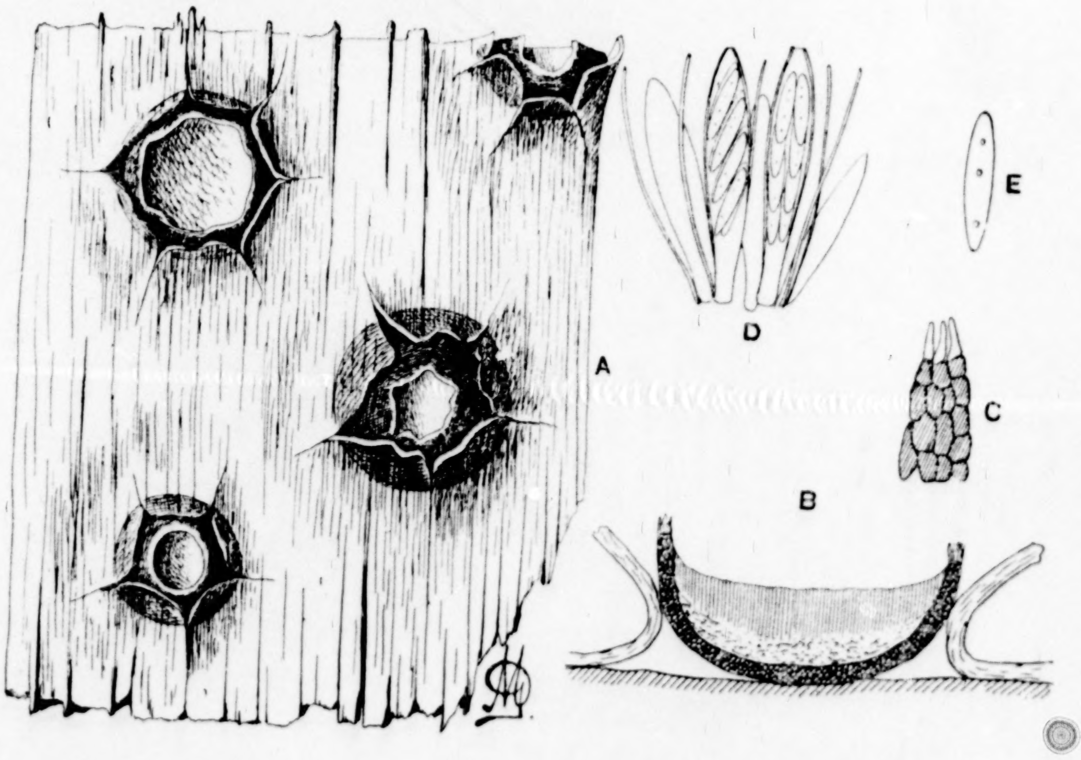
Septoria Rajkoffii Bubák et Moesz



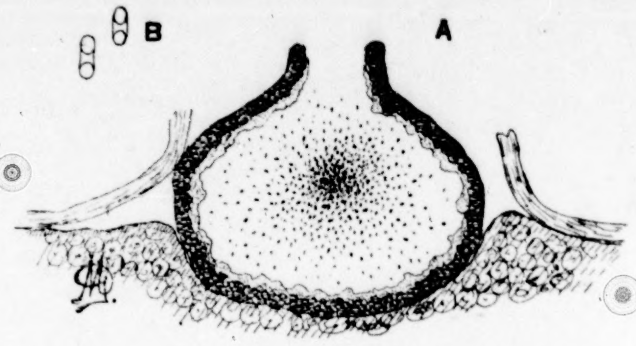
Phaeochora sphaerotheca (Earle) Moesz



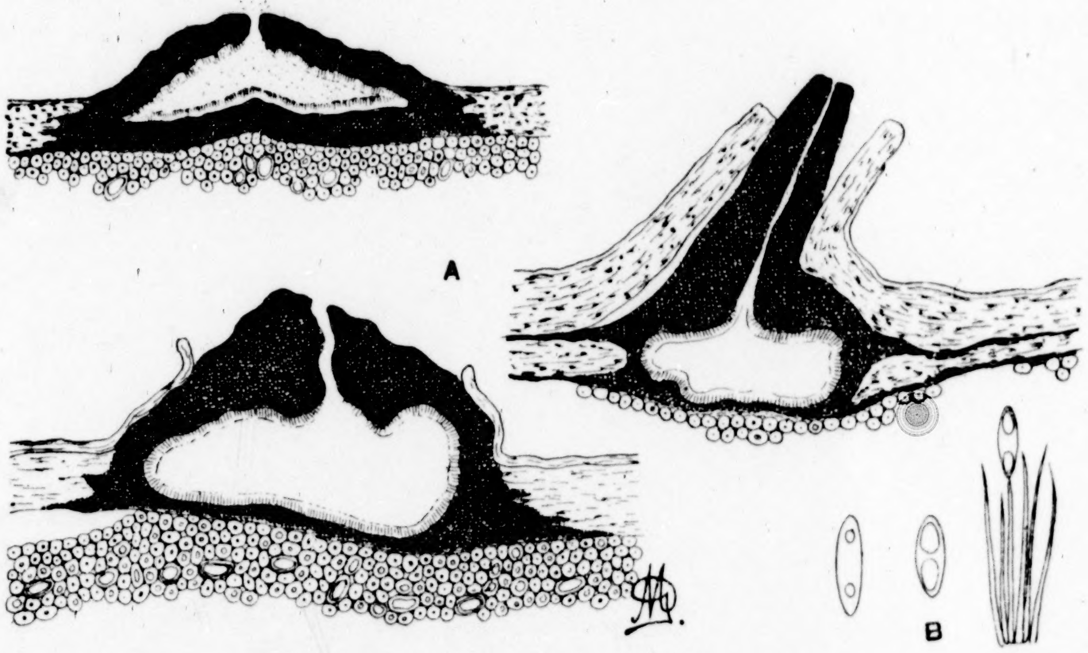
Plectophomella visci (Sacc.) Moesz



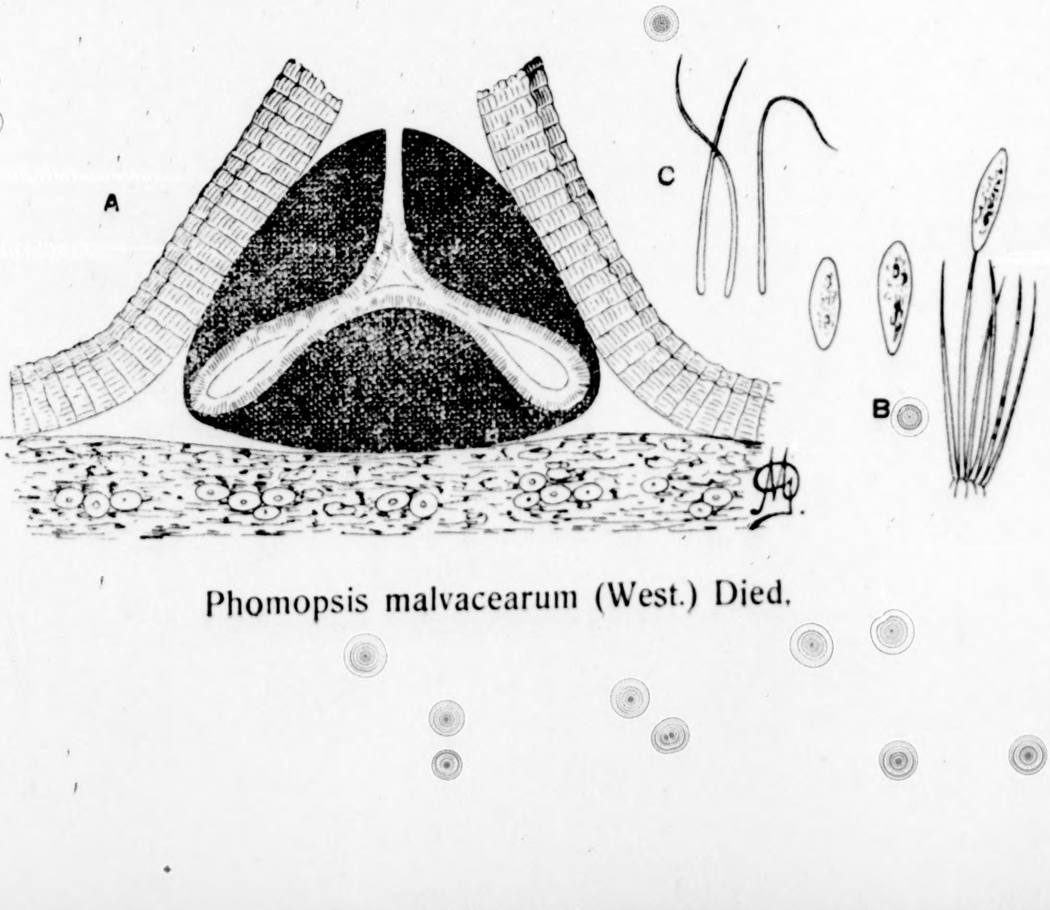
Pyrenopeziza lini Moesz



Sclerophomella harmalae Moesz



Phomopsis dulcamarae (Sacc.) Trev.



Phomopsis malvacearum (West.) Died.

ное угнетение роста его всходов и корневой системы молодых растений. Выделения вяза, как и при обработке дуба и акации, вызывают рост корней второго и третьего порядков у абрикоса, угнетая его рост в высоту.

Из изложенного следует, что корневые выделения испытанных пород стимулируют прорастание семян и первоначальный рост всходов, что хорошо видно на рисунках. Затем они начинают угнетать рост дуба, акации белой и особенно абрикоса. Корневые выделения уничтожают микоризу на корнях дуба и клубеньковые бактерии акации белой. С увеличением количества обработок (поливов) корневыми выделениями у молодых растений исследованных пород уменьшается прирост в высоту, хотя по общей высоте они могут превышать контрольные сеянцы в силу более интенсивного роста в первую половину вегетационного периода.

SUMMARY

The results of laboratory studies showed that the secretions of roots of some tree species stimulate the germination of seed and the emergence of seedlings. Later it is followed by the inhibition of seedling growth observed in English oak, false acacia and especially in apricot; the mycorrhiza in oak roots and nodule bacteria in acacia desappear.

ZUSAMMENFASSUNG

Die Ergebnisse der Laborforschungen haben gezeigt, dass Wurzelausscheidungen bei einigen Holzgewächsen das Aufkommen von Samen und das Wachstum in Anfangsstadium stimulieren. Später wird das Wachstum bei Stieleiche, weisser Robinie und besonders beim Aprikosenbaum gehemmt, dabei verschwinden Knollenbakterien bei weisser Robinie und Mykorhiza auf den Eichenwurzeln.

RESUME

Les résultats des recherches de laboratoire ont montré que les sécrétions de racine de certaines espèces de bois améliorent la germination des semences et la croissance initiale des levées. Puis on observe le ralentissement de la croissance des levées chez le chêne pétiolaire, le faux acacia et surtout l'abricotier. Il faut noter que le mycorhize sur les racines du chêne et les bactéries des nodules chez le faux acacia disparaissent.

труды научных учреждений

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Вопросы механизации животноводческих ферм, техническое обслуживание машинно-тракторных станций.

О НАСЛЕДОВАНИИ МИЛЬДЬЮУСТОЙЧИВОСТИ У ВИНОГРАДА

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УДК 634.87 : 631.52

РАБОТ, посвященных изучению генетических основ селекции винограда на мильдьюустойчивость, очень мало. Поэтому в 1962 и 1963 гг. мы занялись в Молдавской ССР изучением наследственной передачи признака мильдьюустойчивости.

Материалом для исследования послужили имеющиеся на коллекционном и селекционном участках Молдавского научно-исследовательского института садоводства, виноградарства и виноделия межвидовые гибриды F_1 европейско-американского, европейско-амурского и американо-амурского происхождения, а также их исходные формы. Сеянцы второго поколения этих гибридов были получены путем самоопыления соцветий гибридов F_1, а также от скрещивания двух кустов одного и того же гибрида (с целью избежать депрессии у инцухтных сеянцев). Кроме того, были получены семена от возвратного скрещивания гибридов F_1 (Фоль белый $\times$ V. riparia Michx) с европейским сортом Фоль белый. Контролем служили сеянцы от самоопыления соцветий как корнесобственных, так и привитых кустов сорта Фоль бланш. Выращивались также сеянцы V. rotundifolia Michx. ($2n = 40$) от свободного опыления. Этот вид взят как наиболее устойчивый к мильдью из рода Vitis.

Исходные формы и гибриды F_1 изучали путем неоднократного искусственного заражения их мильдью в течение вегетации. Собранные в 1962 г. семена для выращивания сеянцев F_2 высевали весной в бумажных парафинированных сосудах и содержали в тепличных условиях; затем в семядольном состоянии они прошли закалку и в конце мая были посажены в школку. Степень их мильдьюустойчивости устанавливали в конце августа, искусственно заражая в пасмурную погоду под полиэтиленовой пленкой. Этот период, очень благоприятный для развития мильдью, совпал с сильной вспышкой болезни и на производственных участках.

Основные показатели степени мильдьюустойчивости листьев сортов, видов и гибридов винограда мы представили на шкале (см. рис. 1), где изображены различные типы проявления симптомов болезни в конце периода стерилизации. Каждый балл обозначает определенную степень миль-

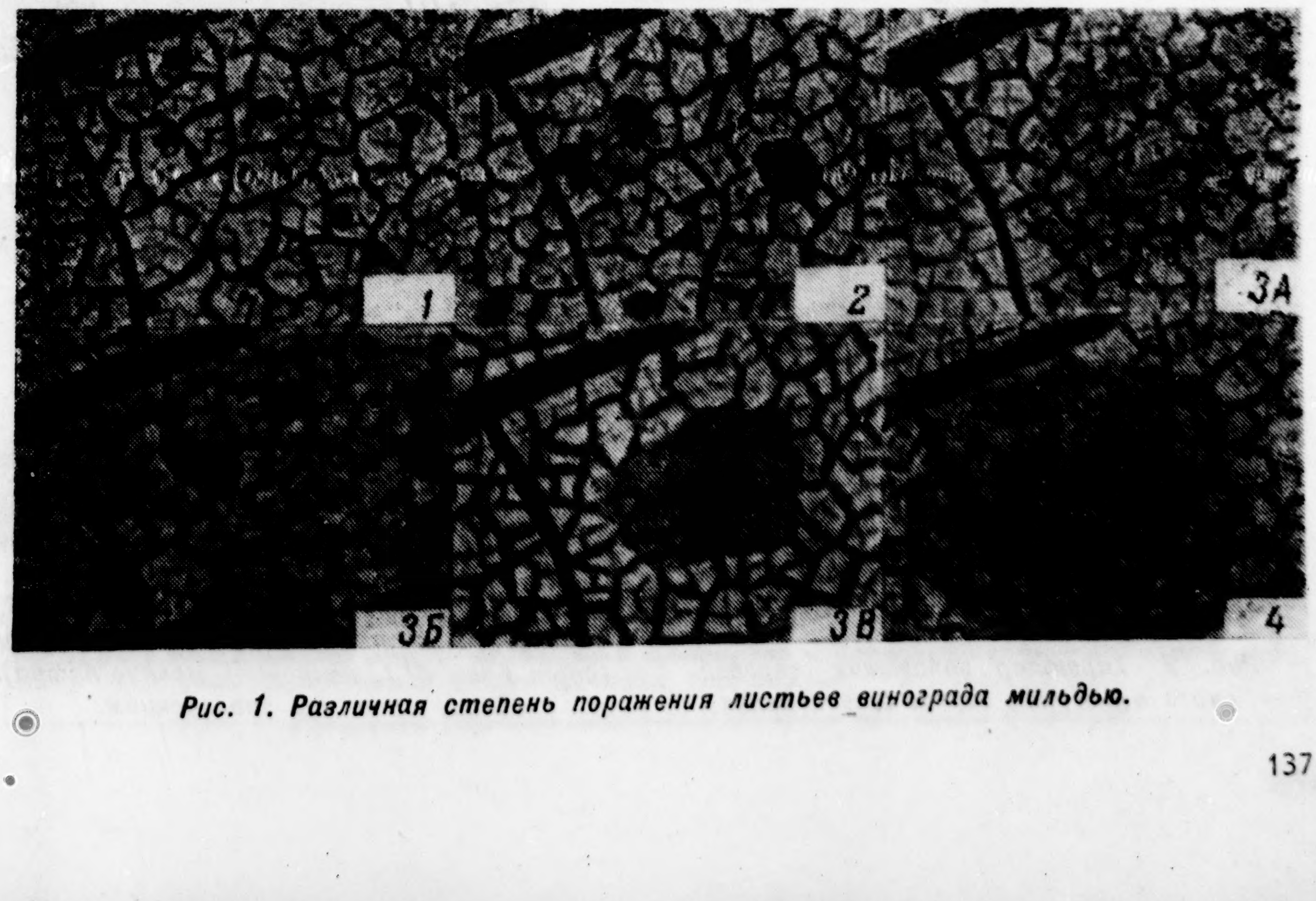

Рис. 1. Различная степень поражения листьев винограда мильдью.

дьюустойчивости, а именно: балл 1 — устьичные и околоустьичные некрозы без распространения мицелия гриба в тканях листа; балл 2 — некрозы, захватывающие площадь листа между мелкими жилками; балл 3А — в местах, где помещены капли суспензии, образуются устьичные и околоустьичные некрозы, но это не препятствует частичному развитию грибницы в месте внесения инфекции, вследствие чего ткань просвечивается. В сухую погоду места развития грибницы высыхают. Если поместить лист во влажную камеру, наблюдается выход негустого спороношения; балл 3Б — мицелий гриба распространяется больше, чем в предыдущем случае, несмотря на образование некрозов, и останавливается только у более прочных жилок. Во влажной камере места поражения покрываются густым спороношением; балл 3В — некрозы при заражении не образуются. Степень распространения мицелия, густота спороношения при высокой влажности воздуха и высыхании зараженных тканей в сухую погоду такие же, как при балле 3А; балл 4 — круглые маслянистые пятна без некрозов. В молодых тканях они сильно увеличены по площади и сливаются. При высокой относительной влажности воздуха образуется очень густое спороношение.

Оценка исходных форм, гибридов первого поколения и сеянцев второго, а также инцухтных сеянцев европейского сорта и сеянцев V. rotundifolia Michx. представлена в таблице.

В результате изучения этого материала установлено, что способность тканей устойчивых видов образовывать некрозы — не основная причина их иммуности. У растений с симптомами болезни, оцениваемыми баллами 3А и 3Б, на листьях хотя и образуются некрозы, но появляются и ограниченные пятна мильдью, которые во влажную погоду дают спороношение гриба. Были также сеянцы F_2, не образующие некрозов, однако, у них появлялись ограниченные пятна мильдью на листьях (балл 3В). Частичное угнетение развития грибницы паразита у этих сеянцев, остановка роста и распространения проросших трубок зооспор после их проникновения в листья можно объяснить токсичностью тканей этих растений по отношению к тканям данного паразита. Такое свойство высокоустойчивых видов совпадает со способностью их образовывать ответные некрозы. Европейские сорта и сеянцы винограда лишены обоих признаков.

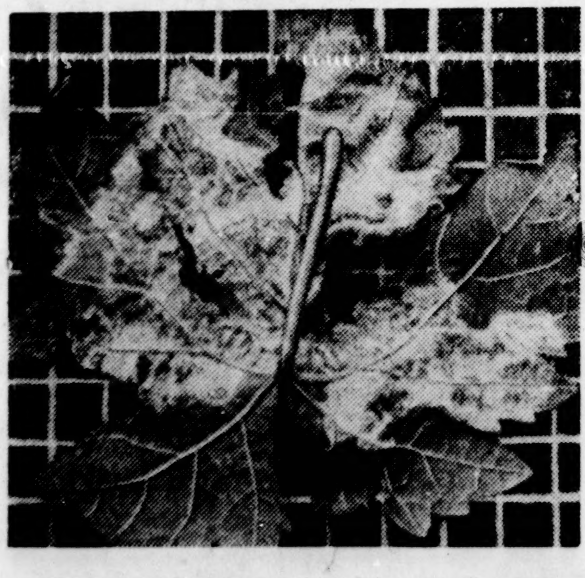

Рис. 2. Характер поражения европейского винограда сорта Шасла белая.

Рис. 3. Проявление мильдью у гибридов F_1 европейско-американского происхождения (сорт Бако № 1, некрозы + мелкие пятна). Внизу лист под увеличением.

Оценка исходных форм, гибридов первого поколения, сеянцев второго поколения и других сеянцев винограда на мильдьюустойчивость

Оценка мильдьюустойчивости (балл): материнской формы	Оценка мильдьюустойчивости (балл): отцовской формы	Оценка гибрида F_1 (балл)	Метод получения сеянцев 2 поколения или европейского сорта	Процент сеянцев с мильдьюустойчивостью, оцениваемой баллом: 1	2	3А	3Б	3В	4	Количество сеянцев в комбинации
V. vinifera L. Фоль белый (корнесобственные кусты)										
4	4	—	Самоопыление	—	—	—	—	—	100,0	36
Фоль белый (привитые кусты)										
4	4		Самоопыление	—	—	—	—	—	100,0	50
V. rotundifolia Michx. (2*n*-40)										
1		—	Свободное опыление	100,0	—	—	—	—	—	15
V. vinifera L. var. Фоль белый × V. riparia Michx. (Бако № 1)										
4	1	3А	Самоопыление	—	9,4	62,5	—	28,1	—	32
V. vinifera L. сорт Фоль белый × V. riparia Michx. (Бако № 1)										
4	1	3А	Скрещивание 2-х кустов этого же гибрида	—	18,4	81,6	—	—	—	38
V. vinifera L. сорт Мускат белый × V. riparia Michx. (Глуар)										
4	1	3А	Самоопыление		—	50,0	21,7	28,3	—	46
V. vinifera L. сорт Мурверд × V. rupestris Sch. (1202)										
4	1	3А	Самоопыление	—	—	68,3	—	31,7	—	41
V. vinifera L. сорт Фоль белый × Фоль белый × ×V. riparia Michx. (Бако № 1)										
4		3А	Возвратное скрещивание		4,6	31,8	—	31,8	31,8	44
V. vinifera L. сорт. Серексия черная (Рара нягрэ) × ×V. amurensis Rupr.										
4		3А	Самоопыление	—	60,9	15,2	—	—	23,9	46
V. vinifera L. сорт. Молдавский (Корно нягрэ) × ×V. amurensis Rupr.										
4		3Б	Самоопыление	—	—	—	19,4	—	80,6	31
V. amurensis Rupr. × Гетш (Мичуринец)										
	—	3А	Самоопыление	—	26,8	63,4	—	—	9,8	41
V. lincecumii Buckl. × V. amurensis Rupr.										
3Б	—	3Б	Самоопыление	—	—	—	—	—	100,0	7

Примечание. В расчет не входило количество карликовых сеянцев с очень короткими междоузлиями и ненормальными листьями. Они обычно не заражаются мильдью

Гибридные растения первого и второго поколений занимают промежуточные места по этим признакам.

Из таблицы видно далее, что оценка гибридов F_1 и расщепляющихся сеянцев второго поколения зависит как от использованных исходных форм, так и от метода получения второго поколения.

В комбинациях европейско-американского происхождения изученные нами гибриды первого поколения — лишь результат вегетативного размножения одного представителя популяции F_1; они образуют ответные некрозы, но вопреки этому ткани их позволяют грибнице паразита развиваться до определенной степени.

Различные методы получения семян от гибридов первого поколения по-разному влияют на признаки образования некрозов и пятен мильдью на листьях сеянцев в потомстве. Разница выражается в характере проявления мильдью и в количестве сеянцев с тем или иным типом поражения этой болезнью.

Некрозы образовывались не у всех самоопыленных сеянцев. Количество тех, которые не образовывали некрозов, зависело от комбинации и составляло 28—31% от общего числа сеянцев в каждой комбинации. У большинства из них были тип листа и пятна мильдью, характерные для гибрида первого поколения. Количество таких сеянцев, например, у второго поколения Мурверд × Рупестрис № 1202 составляло 100% (у 68,3% отмечены некрозы, у 31,7% их не было); отклонения сеянцев второго поколения по степени мильдьюустойчивости от гибрида первого поколения были у комбинации Фоль белый × V. riparia Michx. (Бако № 1). Так, 9,4% сеянцев оказались устойчивее, чем сам гибрид F_1, но они не достигли устойчивости отцовской исходной формы V. riparia Michx. У комбинации Мускат белый × Рипария Глуар часть сеянцев (21,7%) второго поколения по степени устойчивости отклонялась от гибрида F_1 в сторону материнской исходной формы (Мускат белый), но они не достигли полной восприимчивости европейского винограда.

Рис. 4. Проявление мильдью, характерное для американских видов. Внизу — лист под увеличением.

От возвратного скрещивания гибрида F_1 Фоль белый × V. riparia Michx. (в качестве отцовской формы) на сорт Фоль белый (в качестве материнской формы) в потомстве получились разные по форме листа и по мильдьюустойчивости сеянцы. Сеянцев F_2, превосходивших по устойчивости гибрид F_1, было меньше, чем при предыдущих методах создания материала второго поколе-

ния. Сеянцев же с восприимчивостью европейского винограда было довольно много.

В комбинациях европейско-амурского происхождения изученные гибриды F_1 также результат вегетативного размножения одного представителя популяции первого поколения. Эти гибриды способны образовывать ответные некрозы, но сильно различаются по признаку ограничения маслянистых пятен. Гибриды амурский дикий × Гетш (селекционера А. Я. Кузмина) и Сереския (Рара нягрэ) × амурский (селекционеров Х. И. Принца и П. Х. Кискина) устойчивее, чем гибриды Молдавский (Корно нягрэ) × × амурский и Ленцекуми × амурский (селекционера А. Н. Добровольского). Это отражается и в потомствах второго поколения (от самоопыления) этих гибридов. Количество сеянцев второго поколения, пораженных в степени, характеризуемой баллом 4, у комбинации Молдавский (Корно нягрэ) × амурский и Ленцекуми × амурский достигло 80—100%, а у амурский × Гетш и Сереския (Рара нягрэ) × амурский составляло от 9,8 до 23,9%. Сеянцы же с устойчивостью, оцениваемой баллом 2, были только у комбинации амурский × Гетш (26,8%) и Сереския (Рара нягрэ) × × амурский (60,9%). Сеянцы F_2 с поражаемостью (балл 3А) дали только комбинации, обладавшие такой же степенью мильдьюустойчивости в первом поколении (то есть амурский × Гетш и Рара нягрэ × амурский).

Расщепление сеянцев в комбинациях с амурским виноградом обнаруживается и по признаку образования ответных некрозов: все сеянцы, пораженные в степени, оцениваемой баллом 4, не образовали этих некрозов.

Все сеянцы инцухта европейского сорта Фоль бланш как от корнесобственных, так и от привитых кустов поражались в степени, оцениваемой баллом 4, сеянцы же вида V. rotundifolia Michx.— баллом 1.

Развитие грибницы мильдью независимо от способности тканей образовывать некрозы было гистологически доказано Д. Бубалем (1959). По его мнению, ограничение распространения мицелия объясняется тем, что в тканях устойчивых особей не хватает соединений, необходимых для питания паразита. Гистологические данные К. Аренса (1929), результаты заражения тканевых культур (Г. Морел, 1948; Д. Бубаль, 1959), исследования проф. Д. Д. Вердеревского и его сотрудников о роли фитонцидов в устойчивости винограда к данному паразиту подтверждают токсичность тканей устойчивых растений к грибу мильдью.

Самостоятельность в расщеплении признаков формы листа и мильдьюустойчивости наблюдал В. Зотов (1935) у сеянцев F_2 комбинации европейско-американского происхождения. В наших опытах это не обнаружилось, так как сеянцы второго поколения от самоопыленных семян гибридов F_1 европейско-американского происхождения не дали расщепления в сторону европейской исходной формы ни по форме листа, ни по мильдьюустойчивости. Подавляющее большинство этих сеянцев соответствовало по этим двум признакам типу гибрида F_1 и только некоторые отклонились в сторону американской формы. Этот факт говорит о сильной консервативности наследования всех признаков у диких американских видов. Только при возвратном скрещивании гибридов F_1 с европейским виноградом эта консервативность ослабляется, что приводит к свободному расщеплению сеянцев по указанным выше признакам. При использовании амурского винограда не наблюдается такой степени консервативности наследования, как у американских видов. Благодаря этому метод самоопыления позволяет получить во втором поколении свободное расщепление и перекомбинацию признаков обеих исходных форм.

Для выведения высококачественных мильдьюустойчивых сортов винограда европейско-американского происхождения не следует пользоваться методом самоопыления или другими скрещиваниями с участием пыльцы и яйцеклетки американского или гибридного происхождения. Скрещивания здесь должны быть направлены в сторону ослабления консервативности свойств диких видов. Этого можно достичь путем повторного скре-

щивания гибридов с сортами вида V. Vinifera L. при строгом отборе потомства по качеству ягод и мильдьюустойчивости. Повторное введение европейских гамет в гибридную исходную форму не ослабляет мильдьюустойчивости всех особей в полученном потомстве.

В настоящее время есть гибриды прямых производителей, которые по качеству ягод и мильдьюустойчивости намного превосходят гибрид первого поколения Бако № 1 (с устойчивостью, оцениваемой баллом 2). Именно эти гибриды и нужно использовать при скрещивании с европейскими сортами.

При отборе селекционных форм на мильдьюустойчивость в Молдавской ССР и близких по климатическим условиям районах виноградарства можно использовать и растения с устойчивостью, оцениваемой баллами 3А и 3Б. Имеющиеся в коллекции Молдавского научно-исследовательского института садоводства, виноградарства и виноделия гибриды, прямые производители и сорта подвоев с такой степенью устойчивости (например, Бако № 1, Мурверд × Рупестрис 1202 и многие другие) ежегодно растут и плодоносят без лечения. Благодаря этому селекционер имеет больше материала для отбора лучших форм по качеству ягод.

Для дальнейшего улучшения гибридов европейско-амурского происхождения ценным является метод самоопыления. Он позволяет, кроме свободного перекомбинирования признаков во втором поколении, получить много сеянцев, более устойчивых, чем гибрид F_1. Это значительно обогащает материал для отбора высококачественных форм.

Различие по степени мильдьюустойчивости между гибридами первого поколения европейско-амурского происхождения, а также их потомством при самоопылении, очевидно, зависит от степени устойчивости разновидности V. amurensis Rupr., использованной при скрещивании.

Литературные данные о передаче по наследству признака мильдьюустойчивости относятся в основном к комбинациям европейско-американского происхождения.

В первом поколении преобладает неустойчивость, а во втором — происходит расщепление с преобладанием неустойчивых форм (Негруль, 1936). В. Гусфельд (1932), например, получил всего 50 устойчивых растений из одного миллиона сеянцев F_2 гибрида Мурведр × Рупестрис № 1202 после восьмикратной инфекции. Причина этого, как доказал В. Зотов (1935),— искусственное заражение в раннем возрасте, когда игнорируют фактор изменчивости биологических признаков в процессе онтогенетического развития сеянцев.

Д. Бубаль (1959) исследовал характер передачи по наследству ответных некрозов и способности тканей листьев угнетать развитие грибницы паразита. Данные этого автора, указывающие на гомозиготность устойчивости американских видов и неустойчивость сортов V. vinifera L., подтверждались и нашими исследованиями с сеянцами V. rotundifolia Michx. и европейского сорта. В первом поколении, по данным Д. Бубаля и нашим, доминирует свойство образования ответных некрозов. По признаку степени развития мицелия в тканях гибриды F_1 занимают промежуточное положение между исходными формами. Во втором поколении, по нашим данным, устойчивость сеянцев, а также и тип расщепления зависят от исходных форм и метода получения этих сеянцев.

Таким образом, наследование мильдьюустойчивости у винограда не подчиняется одному правилу. Здесь, как указывает А. М. Негруль (1936), в зависимости от исходных форм даже разные скрещивания одной и той же комбинации дают неодинаковый характер устойчивости в F_1 и тип расщепления в F_2. Поэтому подбор пар для селекции на мильдьюустойчивость приобретает важнейшее значение.

SUMMARY

The inheritance of mildew-resistance in grapes is not a regular phenomenon. That's why the selection of components for crosses is of great importance.

When plant breeders use grape varieties of the European-American origin they must not involve self-pollination or other crossing methods which are connected with the presence of pollen or zygotes of the American or hybrid origin. Crossing should be aimed at weakening the conservatism of the nature of wild species.

There are hybrids which are more mildew-resistant than the hybrid of the first generation Baco N 1 which has been given two scores. These hybrids should be used for crossing with European varieties.

Hybrids of the European-Amur origin can be improved by self-pollination.

ZUSAMMENFASSUNG

Die Vererbung der Mehltauresistenz der Weinrebe ist kenerlei Regel unterworfen. Daher gewinnt die Auswahl des Elternpaars für die Zucht große Bedeutung.

Dienen als Selektionsgut Weinrebensorten europäischamerikanischen Ursprungs, soll man sich nicht der Methode der Selbstbefruchtung oder andrer Kreuzungen unter Anteilnahme des Pollens und der Eizelle amerikanischer oder hybridischer Abkunft bedienen. Die Kreuzungen hier müssen gerichtet sein im Sinne einer Abschwächung des Konservativismus der Eigenschaften der wilden Arten.

Es gibt Hybriden, welche nach der Mehltauresistenzfähigkeit dan Bastard der ersten Generation der Sorte Bako No 1, welche bei der Bewertung die Note 2 erhielt, um ein Bedeutendes übersteigen. Diese sind für die Kreuzung mit europäischen Sorten zu verwenden.

Für die Verbesserung der Hybriden amureuropäischer Herkunft kann die Methode der Selbstbefruchtung genutzt werden.

RESUME

L'hérédité de la résistance de la vigne au mildiou n'est pas soumise à une règle. C'est pourquoi le choix des paires pour la sélection a une grande importance.

En utilisant les variétés de la vigne d'origine européenne-américaine il ne convient pas d'appliquer la méthode d'autofécondation ou d'autres croisements en présence du pollen et de l'oosphère d'origine américaine ou hybride. En ce cas les croisements doivent être réalisés dans le sens de l'affaiblissement du conservatisme des propriétés des espèces sauvages.

Il y a des hybrides qui, d'après leur résistance au mildiou, dépassent de beaucoup l'hybride de F_1 de Bako N 1 qui a reçu une note égale à deux points. Il faut les croiser avec les variétés européennes.

Pour améliorer les hybrides d'origine européenne-d'Amour on peut utiliser la méthode d'autofécondation.

труды научных учреждений

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Inactivation of Southern Bean Mosaic Virus by Ultraviolet Light[1]

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Inactivation of Southern bean mosaic virus by ultraviolet light (wavelength 253.7 mμ) was found to follow the course of a first-order reaction for at least 3 log units. The rate of inactivation was dependent upon the initial concentration of virus, being 3.7 times as fast for a solution containing 0.1 mg/ml of virus as for one containing 1.0 mg/ml. Evidence was obtained that the inactivating effect of ultraviolet light could be partially reversed by visible light; the rate of inactivation of a solution containing 1.0 mg/ml of virus was 1.5 times as fast when assay plants were kept for a few hours in darkness immediately after they were inoculated as when the assay plants were kept in the light for the first few hours after inoculation.

INTRODUCTION

Very little research has been done on the effects of either ionizing or nonionizing radiation on the so-called small spherical viruses, although rather extensive studies have been made on the effects of radiation on tobacco mosaic virus. The subject matter has been reviewed in several places (Pollard, 1954; Kleczkowski, 1957; Price, 1964). The effect of deuterons on Southern bean mosaic virus (SBMV) was studied to some extent by Pollard and Dimond (Pollard, 1954). Bawden and Kleczkowski (1953) reported that inactivation of tomato bushy stunt virus (TBSV) and tobacco necrosis virus (TNV) proceeded according to a first-order reaction when the viruses were exposed to light of wavelength 253.6 mμ.

Investigation of the effects of radiation on Southern bean mosaic virus, a virus with an essentially spherical particle and a diameter of about 25 mμ (Miller and Price, 1946a), would seem to be of value in contributing to knowledge about the mechanism of action of radiation. The present paper is concerned with the rates of inactivation of SBMV by radiation of wavelength 253.7 mμ and with photoreactivation of partially inactivated virus.

MATERIALS AND METHODS

Virus Preparations

The source of the Southern bean mosaic virus used was Isolate no. AC-17 of the American Type Culture Collection of plant viruses. It was propagated in plants of the Bountiful variety of garden bean (*Phaseolus vulgaris* L.), which were harvested about 3 weeks after being inoculated.

Purified preparations were obtained by centrifuging sap of infected bean plants alternately at 10,000 rpm for 10 minutes and at 29,000 rpm for 90 minutes in the no. 30 rotor of a Spinco model L centrifuge (Price, 1946). Pellets from the high speed run were dissolved in distilled water so as to obtain essentially salt-free preparations. The number of cycles of alternating low speed-high speed centrifugation required to obtain virus preparations relatively free of impurities varied from one lot of bean plants to another. Three cycles were used in the present work.

The ultraviolet (UV) absorption spectrum

[1] Florida Agricultural Experiment Stations Journal Series no. 1893. This investigation was supported in part by research grant AI 03148 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, United States Public Health Service.

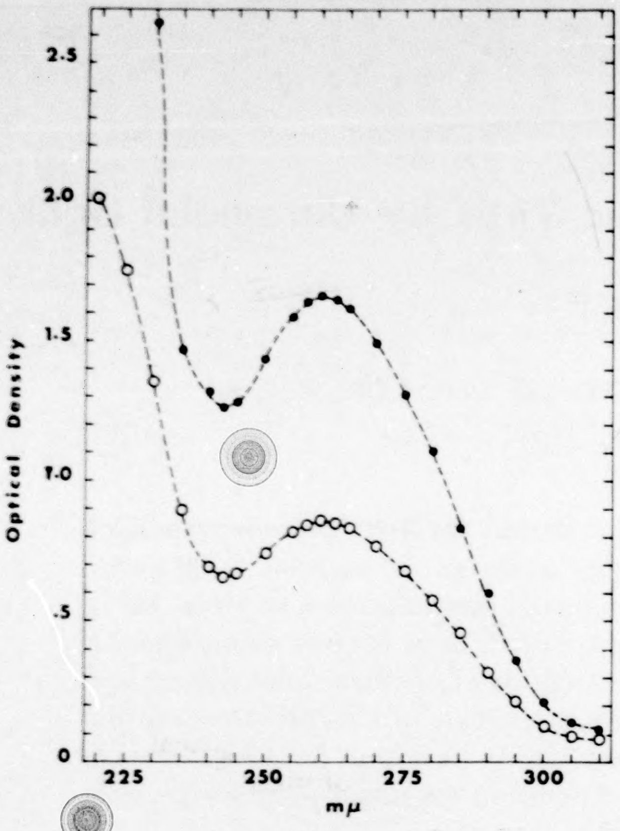

FIG. 1. Ultraviolet light absorption spectrum of Southern bean mosaic virus. Upper curve 0.315 mg/ml; lower curve 0.158 mg/ml.

of two dilutions of a purified preparation of SBMV is given in Fig. 1; absorption was measured with a Zeiss PMQ II spectrophotometer. The ratio of the optical density at the peak (260 mμ) to that at the valley (242.5 mμ) is about 1.3. Ratios have varied from about 1.2 to 1.4 in different preparations of SBMV.

The shape of the UV absorption curve is an indication of the purity of the virus preparation. Preparations that absorb UV in the manner indicated by Magdoff's (1960) Fig. 2,A can be further purified by allowing them to stand at 4°C for a few days and then subjecting them to an additional cycle of high speed-low speed centrifugation.

The purified preparations were stored at 4°C. A small amount of a 1:1000 dilution of merthiolate was added as a preservative.

Determination of Virus Concentration

The virus concentration of the preparations used was determined by absorbency at a wavelength of 260 mμ, as suggested for TMV by Takahashi (1951). In preliminary studies it was found that the absorbency of purified preparations of SBMV is a linear function of concentration between the limits of 0.025 and 0.45 mg/ml (Fig. 2); in these preliminary experiments, the concentration of 4 preparations of SBMV was initially determined by dry weight and appropriate dilutions for readings in the Zeiss spectrophotometer were made from them in distilled water. The relationship can be expressed as follows:

$$\text{Concentration (mg/ml)} = 0.185 \text{ O.D.} \quad (\text{at } 260 \text{ m}\mu)$$

Irradiation Procedure

The ultraviolet light source was a Spectroline SL 2537 lamp with a filter that transmits a high percentage of radiation of 253.7 mμ. According to the manufacturer, the lamp delivers 40 μw/cm² at a distance of 18 inches with the filter in place; this is equivalent to 316 μw/cm² at a distance of 16 cm.

One milliliter of the virus solution to be irradiated was placed in an aluminum saucer and was continuously stirred by means of a magnetic stirrer during irradiation. All samples were irradiated at a distance of 16 cm from the source. The irradiated sample was then diluted to an appropriate concentration with 0.01 *M* phosphate buffer at pH 7 for assay.

Assays

The method used for determining the relative activity of a virus solution after treatment with ultraviolet light has been described elsewhere in detail (Price, 1945; Price and Holt, 1948). In essence, the method consists in comparing two dilutions of the treated sample, U1 and U2, with two dilutions of a standard, S1 and S2, the standard in this case being the nonirradiated virus. The dilution interval was always 1:10 and the two dilutions were selected to give an appropriate number of lesions when the samples were inoculated onto the primary leaves of Kentucky Wonder Wax bean. Each of the 4 virus samples was tested on 24 half-leaves.

Lesions were counted 5–7 days after the plants were inoculated. Since the variance of the numbers of lesions produced is a percentage of the mean number, the individual lesion counts were converted to logarithms, the number 1 being added to each count (Bliss and Calhoun, 1954). The logarithms

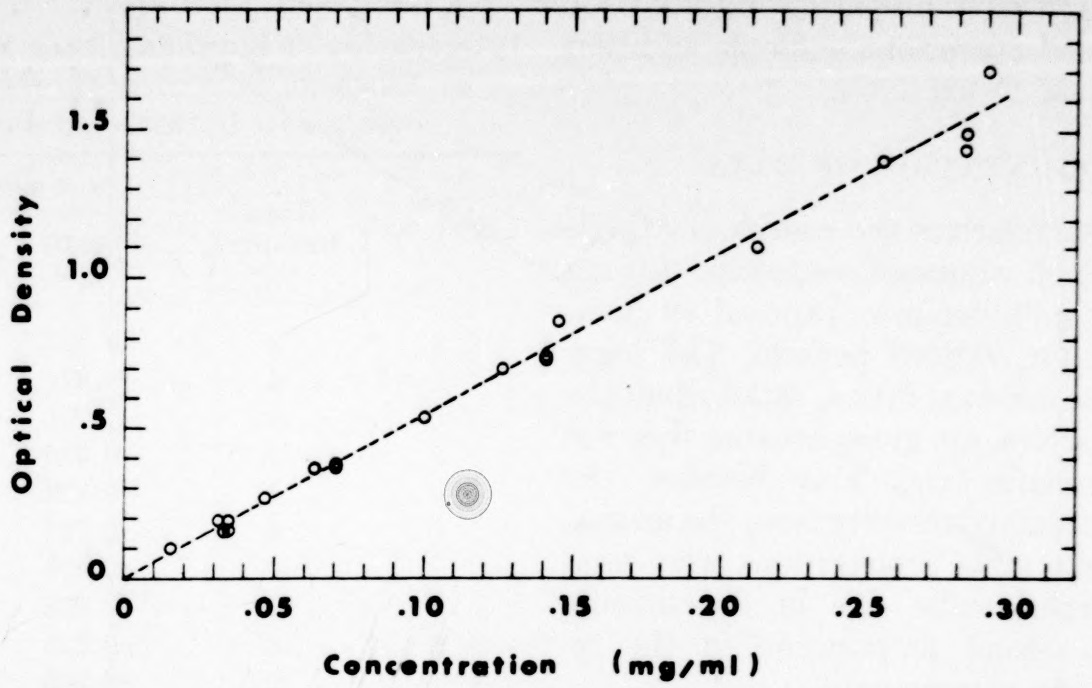

FIG. 2. Regression of concentration of SBMV (determined by dry weight) on optical density at 260 mμ. The points represent experimental data for four different preparations of virus.

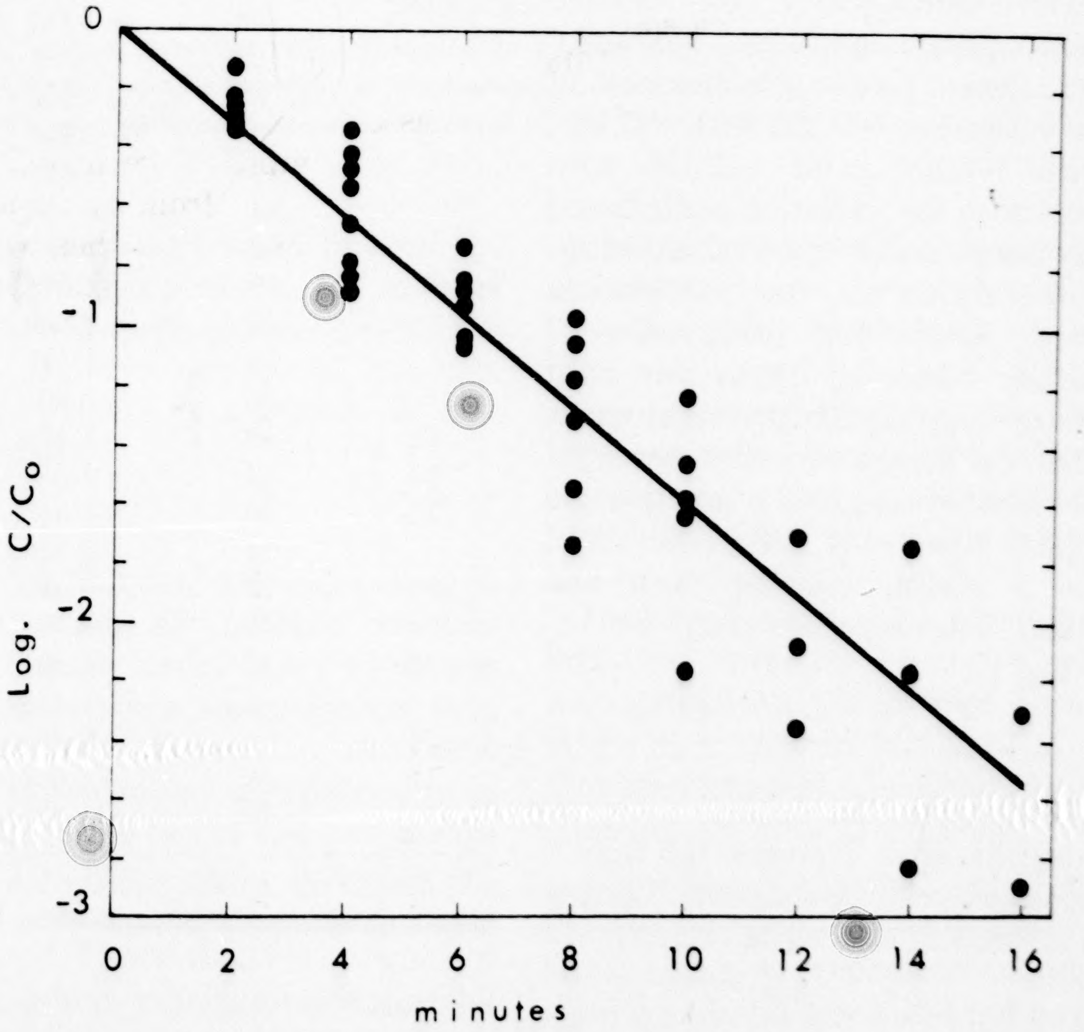

FIG. 3. Logarithm of survival ratios of Table 1 plotted as a function of time. The straight line represents the rate of inactivation of virus in samples containing 1.0 mg/ml.

were then totaled to give a sum for each of the 4 virus samples, U1, U2, S1, and S2. The log ratio of potencies was computed from these 4 totals according to the equation $M = dI/b$, where $d = (U1 + U2) - (S1 + S2)$, $b = (U1 + S1) - (U2 + S2)$, and I, the interval in logarithms between dilutions, is equal to 1. The log of the survival ratio, $\log C/C_0$, is the same as M except when different dilution factors were

used for S and U in preparing solutions for assay; in these exceptions a simple addition will convert M to $\log C/C_0$.

PRESENTATION OF DATA

Table 1 summarizes the results of experiments in which solutions containing 1.0 mg of virus per milliliter were exposed to ultraviolet light for various periods. The logarithms of the survival ratios, rather than the ratios themselves, are given because they are more meaningful and also because the figures are obtained directly from the assays. Two different virus preparations were used in these experiments, one in experiments 2–5 and a second preparation in the remainder of the experiments.

In experiment 7, solutions that had been exposed for 2, 6, and 8 minutes, respectively, were assayed concurrently on Golden Cluster bean plants and on Kentucky Wonder Wax bean plants. Comparison of the log survival ratios, −0.265 with −0.133, −0.873 with −0.837, and −1.310 with −1.535, illustrates the variation contributed by the assay itself, rather than variation encountered in carrying out the inactivation.

It has been shown that inactivation of tobacco mosaic virus by ultraviolet light follows a simple exponential survival curve equivalent to the course of a first-order reaction (Price and Gowen, 1937). Inactivation of other plant viruses by ultraviolet light proceeds in a similar manner (Lea and Smith, 1940). The reaction may be expressed by Eq. 1,

$$\ln (C/C_0) = kt \qquad (1)$$

in which C_0 is the initial concentration, C the concentration after exposure for time t, and k the reaction velocity constant. Letting $k' = 0.4343k$, we can also write (Eq. 2)

$$\log C/C_0 = k't \qquad (2)$$

The log survival ratios determined by assays in which test plants were kept in the light for 4 or more hours after inoculation are plotted in Fig. 3 as a function of time of exposure in minutes. The straight line of Fig. 3 was fitted to the experimental points so as to pass through the origin by means of the relation $k' = Sxy/Sx^2$, in which x represents time in minutes and y represents $\log C/C_0$.

TABLE I

INACTIVATION OF SOUTHERN BEAN MOSAIC VIRUS BY EXPOSING SOLUTIONS CONTAINING 1.0 MG/ML TO ULTRAVIOLET LIGHT[a]

Expt. no.	Time (minutes)	Log of survival ratio: Assayed in light	Log of survival ratio: Assayed in dark
2	2	−0.323	
	4	−0.824	
	8	−1.051	
3	2	−0.326	
	4	−0.879	
	6	−0.722	
	8	−0.925	
5	4	−0.658	
6	4	−0.336	
7	2	−0.265	
	4	−0.530	
	6	−0.873	
	8	−1.310	
	2*	−0.133	
	6*	−0.837	
	8*	−1.535	
9	10	−1.633	
	12	−2.075	
	14	−2.156	
	16	−2.884	−4.756
10	10	−2.148	
	12	−1.699	
	14	−1.735	
	16	−2.297	−3.169
11	10	−1.573	
12	12	−2.352	−2.786
13	10	−1.447	−2.393
	14	−2.829	−4.033
14	2	−0.230	−0.468
	4	−0.780	−0.957
15	6	−0.924	−1.486
	8	−1.173	−1.594
16	4	−0.466	−0.941
17	6	−1.031	−1.411
	10	−1.223	−2.151

[a] Assays on Golden Cluster bean plants; other assays on Kentucky Wonder Wax bean plants.

Under the conditions of the experiments, inactivation of SBMV by ultraviolet light is, therefore, represented by the equation

$$\log C/C_0 = -0.159 \pm 0.010 \text{ minutes}^{-1}$$

at the 5% level of significance.

It is apparent from Fig. 3 that inactivation

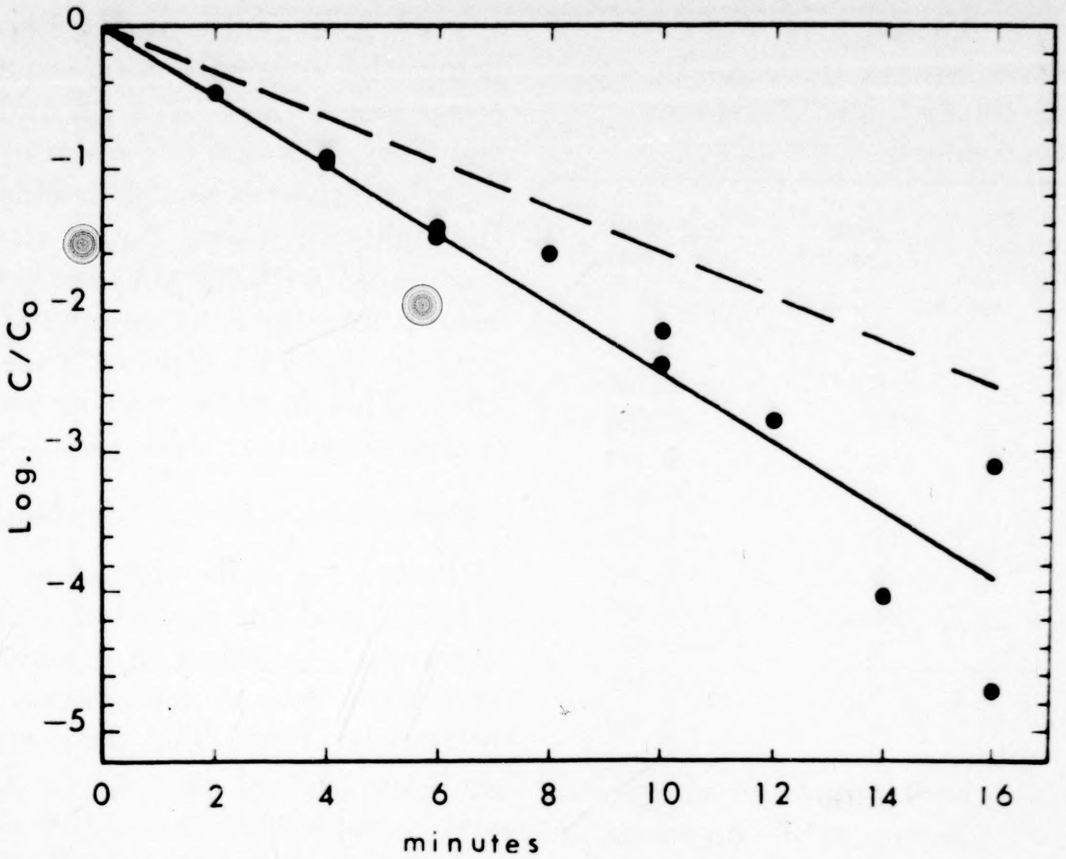

FIG. 4. Logarithm of survival ratios from Table 1 when assays were made on plants kept for a few hours in darkness plotted as a function of time of exposure to ultraviolet light. The solid line was fitted by the method of least squares to the experimental points. The dash line is the same as the solid line in Fig. 3.

of SBMV by ultraviolet light follows the course of a first-order reaction for at least 3 log units. Although inactivation was 99.7% complete, on the average, in 16 minutes, there was no evidence of a change in rate during the period.

Photoreactivation

According to Bawden and Kleczkowski (1953), inactivation of tobacco necrosis virus and of tomato bushy stunt virus was about 20% faster when assay plants were kept in the dark than when they were kept in the light. Subsequently, this difference in rate was considered to be the result of photoreactivation (Bawden and Kleczkowski, 1955), six of seven viruses tested being photoreactivated. Photoreactivation was not demonstrated when irradiated virus itself was exposed to light, but only when irradiated virus was used to inoculate a plant and the inoculated plant was then kept in the light.

Experiments were carried out to determine whether or not irradiated SBMV could be photoreactivated. Two groups of assay plants were inoculated with virus that had been irradiated; one group was kept on the greenhouse bench under normal conditions; the other group was kept on the same bench but was covered with a light-tight box within ½ hour after being inoculated and kept covered during the afternoon and evening of the day on which the plants were inoculated. Data from these experiments are also presented in Table 1.

In each instance, the computed survival ratio was lower for irradiated samples that were assayed on plants kept for a few hours in the dark than for the same samples when they were assayed on plants kept in the light immediately after inoculation.

The logarithm of survival ratios are plotted as a function of time in Fig. 4. The solid line in this figure represents the rate of survival when samples were assayed on plants kept in darkness whereas the dash line, which is equivalent to the solid line of Fig. 3, represents the rate of survival when samples were assayed on plants kept in the light. The equation for survival in the former case is $\log C/C_0 = -0.246 \pm 0.028$ minutes^{-1} at the 5% level of significance, which may be

TABLE 2

INACTIVATION OF SOUTHERN BEAN MOSAIC VIRUS BY ULTRAVIOLET LIGHT. EFFECT OF INITIAL VIRUS CONCENTRATION

Expt. no.	Concentration (mg/ml)	Time (minutes)	Log of survival ratio
18	0.5	4	−1.565
		8	−2.105
		12	−3.680
19	2.0	6	−0.494
		12	−0.918
		17	−1.524
		23	−2.263
		28	−2.896
20	0.5	2	−0.659
		4	−1.801
		6	−2.180
		8	−1.545
21	0.1	0.42	−0.372
		0.83	−0.446
		1.25	−0.814
		1.67	−0.970
		2.08	−1.220
22	0.1	0.42	−0.217
		0.83	−0.425
		1.25	−0.769
		1.67	−1.084
		2.08	−1.043
23	0.5	2	−0.477
		4	−1.192
		6	−1.498
		8	−1.861
		10	−2.374

compared with $\log C/C_0 = -0.159 \pm 0.010$ minutes^{-1} for assay plants kept in the light.

The difference between the two velocity constants is highly significant. It may be interpreted to mean that SBMV inactivated by exposure to ultraviolet light can be partially photoreactivated after assay plants are inoculated with it.

In the 12 duplicate assays with plants in light versus plants in dark, there was observed a small but consistent difference in the number of lesions produced by the standard virus preparation. The difference was shown to be highly significant by analysis of variance. The mean number of lesions per half-leaf on 288 half-leaves for plants in the dark was 15.9, and it was 18.2 for plants in the light. These figures were obtained by means of the equation $\bar{v} = \text{antilog}(\bar{y} + 1.15 s_y^2) - K/2$ (Bliss and Calhoun, 1954), v being the original lesion counts, y being equal to $v + K/2$, $K = 2$, and s_y being the standard deviation of y obtained from analysis of variance. Keeping inoculated plants in the light for a few hours after they were inoculated with SBMV increased the number of infections as compared with plants kept in the dark, the increase being about 16%. This increase was in addition to the increase resulting from photoreactivation.

Effect of Initial Virus Concentration

Tests were made with solutions containing 0.1, 0.5, and 2.0 mg/ml of virus in order to determine the effect of initial virus concentration on rate of inactivation of SBMV by ultraviolet light. The data are in Table 2 and are plotted in Fig. 5. The rate constants calculated from the data are 1.34 min^{-1} for 0.1 mg/ml, 0.632 min^{-1} for 0.5 mg/ml, and 0.213 min^{-1} for 2.0 mg/ml. They may be compared with 0.367 min^{-1}, the constant previously calculated for 1.0 mg/ml of virus.

It was known as early as 1927 (Fisher and McKinley, 1927) that inactivation of viruses by ultraviolet light is influenced by the initial concentration of virus. This influence has been discussed in detail by McLaren (1949, 1951) and by Oster and McLaren (1950). The relationship is expressed by the equation

$$k = \frac{\phi I_{\text{abs}}}{C_0} \qquad (3)$$

in which k is the rate constant, C_0 is the initial concentration, I_{abs} is the intensity of the radiation absorbed, and ϕ is the quantum yield. The quantum yield may be defined as the ratio of the number of virus particles inactivated to the number of photons of light absorbed. The quantum yield is generally considered to be a constant over a wide range of virus concentrations.

From Eq. 3, the rate of inactivation of SBMV is expected to be inversely proportional to the initial concentration of virus. But I_{abs}, the intensity of the radiation absorbed, is itself related to C_0, the initial virus concentration. Equation 3 can be written $kC_0 = \phi I_{\text{abs}}$. The values obtained

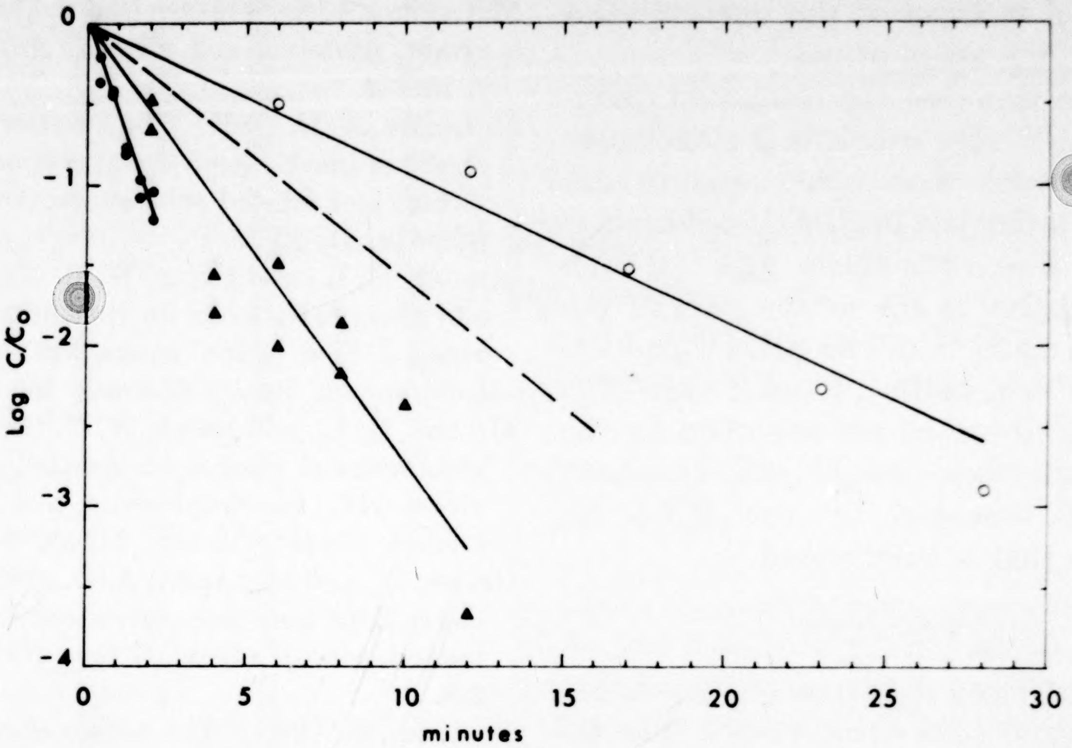

FIG. 5. Logarithm of survival ratios from Table 2 for various concentrations of SBMV plotted as a function of time in minutes. Concentrations, from top, reading clockwise, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml, and 0.1 mg/ml.

for kC_0 are 0.134, 0.316, 0.367, and 0.426 when C_0 equals 0.1, 0.5, 1.0, and 2.0 mg/ml of SBMV, respectively. Thus, the intensity of the radiation absorbed increased greatly as the concentration of virus was increased from 0.1 to 0.5 mg/ml and somewhat less rapidly as the concentration was increased from 0.5 to 2.0 mg/ml. In the experiments reported, no attempt was made to measure the intensity of the radiation absorbed.

Bawden and Kleczkowski (1953) found the rate constants for inactivation of TMV and TNV to be much less for concentrated solutions than for dilute solutions and interpreted this to mean that less radiation was absorbed in unit time per virus particle when more particles were present. They did not measure the intensity of radiation absorbed, nor did they calculate a quantum yield.

The Quantum Yield

By making certain assumptions, it is possible to calculate a quantum yield for inactivation of SBMV by ultraviolet light. According to Oster and McLaren (1950), the quantum yield for inactivation is given by

$$\phi = \frac{Cf}{MEF} \qquad (4)$$

in which C is the concentration in grams per milliliter, f is the fraction of virus inactivated during the time of exposure, M is the molecular weight, E is the einsteins falling on 1 ml of the solution during exposure, and F is the fraction of light absorbed by active virus during exposure. Oster and McLaren estimated that 64% of the light of 253.7 mμ falling on a solution that contained 6.4×10^{-4} g/ml of TMV was actually absorbed by the virus and used this percentage to calculate a quantum yield of 4.3×10^{-4} for TMV. It can be assumed that 64% of the radiation applied to 1 mg/ml of SBMV in the experiments reported above was absorbed by active virus, that the surface area of the solution being exposed to the light was 1.0 cm², and that the lamp delivered 316 μw/cm², as stated by the manufacturer, or 4.53×10^{-6} einsteins/min. The molecular weight of SBMV is 6.6×10^6 (Miller and Price, 1946a). When the rate constant -0.246 is used.

$$\phi = \frac{0.43 \times 10^{-3}}{6.6 \times 10^6 \times 4.53 \times 10^{-6} \times 0.64} = 2.25 \times 10^{-5}$$

This quantum yield is about 1/10 that obtained by Oster and McLaren (1950) for TMV but should be regarded as only an

approximation because of the uncertainties in the underlying assumptions.

Despite the fact that the computed quantum yield is not very exact, it is sufficiently exact to indicate that many quanta are absorbed by a particle of SBMV before it is inactivated. The ribonucleic acid (RNA), which presumably is the active part of the virus, makes up 21% of the SBMV particle (Miller and Price, 1946b). Even if only 21% of the quanta absorbed are absorbed by the RNA fraction, there would still be about 9000 quanta absorbed by the RNA for each particle that is inactivated.

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KUHN, C.W. (Ga Exp. Stn, Experiment.)
Decline of specific infectivity of Cowpea chlorotic mottle virus in vivo.
Virology, 25 (1):9-14, 6 graphs, 1 table, 1965.
[14 ref.]
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...ctivity of Cowpea Chlorotic ...rus *in Vivo*[1,2]

... KUHN

...*Experiment Station, Experiment, Georgia*

...*ptember 10, 1964*

...ic mottle virus (CCMV) declined with ad-
...plants and inoculated primary leaves. The
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In vivo inactivation of plant viruses has been recognized for many years. Partial recovery from severe symptoms with increasing length of infection is common to several plant virus diseases (Bawden, 1964), and in some cases the recovery is accompanied by a decline in virus nucleoprotein concentration (Price, 1936; Ross, 1941). Studies concerning specific infectivity, the amount of biological activity per specific unit of virus, offer a means of following biological changes that occur during an infection period. Results presented here indicate that CCMV loses infectivity *in vivo* without denaturation of its nucleoprotein.

MATERIALS AND METHODS

The virus was cultivated in cowpea, *Vigna sinensis* (L.) Savi var. Early Ramshorn and Monarch, and harvests were made 1–55 days after inoculation. The inoculum was cowpea leaves ground in 0.01 *M* neutral phosphate buffer and 1% Celite. To avoid the possibility of a strain producing a unique biological phenomenon, numerous single-lesion isolates were used throughout the study with similar results.

The virus was purified by extraction with *n*-butanol and chloroform, 2 or 3 cycles of ultracentrifugation, and rate or equilibrium density gradient centrifugation (Kuhn, 1964). This treatment produced a highly purified virus sample as recognized by the homogeneous particle size (about 32 mμ), a single component moving in a density gradient column, reproducible phosphorus values, and a distinctive ultraviolet absorption curve (Kuhn, 1964). All specific infectivity comparisons were made with purified virus material unless otherwise stated.

Infectivity assays were conducted on primary leaves of soybean, *Glycine max* (L.) Merr. var. CNS-4. Local-lesion comparisons were made on a half-leaf basis in an incomplete block design with treatment replications ranging from 6 to 12. Although the useful lesion number range was 15–200 per half-leaf, the most reliable assays were obtained with counts below 85. Larger numbers than 85, however, were used and were acceptable because of large differences between some treatments. Where large differences occurred, 2 or 3 comparable concentrations of each treatment were assayed, in the same or separate assays, and the average is reported

[1] This investigation was supported in part by grant GM 12039-01 from the National Institutes of Health, United States Public Health Service.

[2] Journal Series Paper No. 479, Georgia Experiment Station, University of Georgia.

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VIROLOGY **25**, 9–14 (1965)

Decline of Specific Infectivity of Cowpea Chlorotic Mottle Virus *in Vivo*[1,2]

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Specific infectivity of cowpea chlorotic mottle virus (CCMV) declined with advancing age of infection in both whole plants and inoculated primary leaves. The decline began between 2 and 3 days after inoculation; 20–30 days later, purified preparations with similar amounts of virus nucleoprotein were only 1–5% as infective as the initial preparations. Specific infectivity was independent of host age, purification procedures, aggregation, and oxidative inhibitors. Specific infectivity increased when infected plants were transferred to 16°, presumably as a result of new particle formation; it declined rapidly at 33°.

INTRODUCTION

In vivo inactivation of plant viruses has been recognized for many years. Partial recovery from severe symptoms with increasing length of infection is common to several plant virus diseases (Bawden, 1964), and in some cases the recovery is accompanied by a decline in virus nucleoprotein concentration (Price, 1936; Ross, 1941). Studies concerning specific infectivity, the amount of biological activity per specific unit of virus, offer a means of following biological changes that occur during an infection period. Results presented here indicate that CCMV loses infectivity *in vivo* without denaturation of its nucleoprotein.

MATERIALS AND METHODS

The virus was cultivated in cowpea, *Vigna sinensis* (L.) Savi var. Early Ramshorn and Monarch, and harvests were made 1–55 days after inoculation. The inoculum was cowpea leaves ground in 0.01 *M* neutral phosphate buffer and 1% Celite. To avoid the possibility of a strain producing a unique biological phenomenon, numerous single-lesion isolates were used throughout the study with similar results.

The virus was purified by extraction with *n*-butanol and chloroform, 2 or 3 cycles of ultracentrifugation, and rate or equilibrium density gradient centrifugation (Kuhn, 1964). This treatment produced a highly purified virus sample as recognized by the homogeneous particle size (about 32 mμ), a single component moving in a density gradient column, reproducible phosphorus values, and a distinctive ultraviolet absorption curve (Kuhn, 1964). All specific infectivity comparisons were made with purified virus material unless otherwise stated.

Infectivity assays were conducted on primary leaves of soybean, *Glycine max* (L.) Merr. var. CNS-4. Local-lesion comparisons were made on a half-leaf basis in an incomplete block design with treatment replications ranging from 6 to 12. Although the useful lesion number range was 15–200 per half-leaf, the most reliable assays were obtained with counts below 85. Larger numbers than 85, however, were used and were acceptable because of large differences between some treatments. Where large differences occurred, 2 or 3 comparable concentrations of each treatment were assayed, in the same or separate assays, and the average is reported

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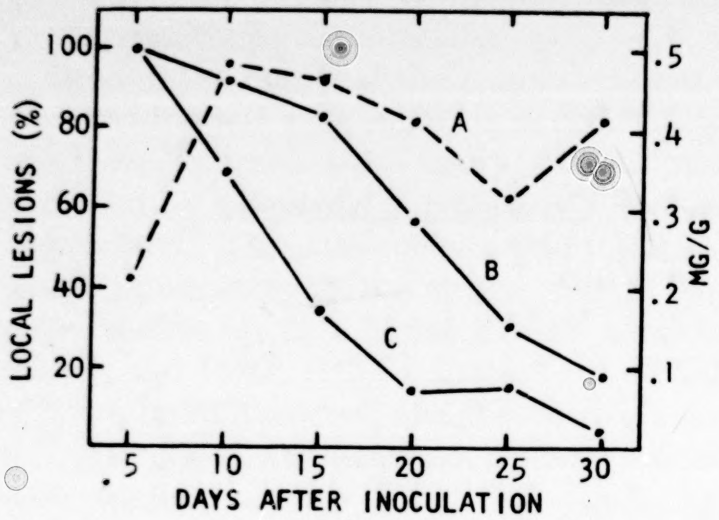

FIG. 1. Virus nucleoprotein concentration (curve *A*, milligrams of virus per gram of plant tissue), sap infectivity (curve *B*, 100% = 146 lesions per half-leaf), and specific infectivity (curve *C*, 100% = 112 lesions per half-leaf) of CCMV grown in whole cowpea plants.

in the results. With smaller differences, treatment dilutions were adjusted to give counts of 85 or below.

In this paper the main basis for specific infectivity comparisons was optical measurements of purified virus samples on a Beckman DU spectrophotometer. Virus samples were adjusted to a specific optical density (±0.002) between 0.040 and 0.400 at 260 mμ. The usual range was 0.040–0.100, but higher concentrations were necessary for virus from old infections and for virus that was frozen and thawed more than once. The major criterion of purity was the 280:260 mμ and 240:260 mμ ratios (0.60–0.61 and 0.69–0.70, respectively) obtained from the ultraviolet absorption curve (Kuhn, 1964). Samples which deviated from these ratios were subjected to further purification or discarded. Virus nucleoprotein concentration was based on ultraviolet absorption of purified virus at 260 mμ correlated with dry weight (0.560 optical density = 0.1 mg/ml). The validity of spectrophotometric equalization was confirmed by serological and phosphorus tests. Virus samples equalized optically at 260 mμ, and with similar 280:260 mμ and 240:260 mμ ratios, had the same serological end point and the same amount of phosphorus. Phosphorus quantities were determined by the Allen method (Allen, 1940). CCMV antiserum was prepared as described previously (Kuhn, 1964), and virus end points were determined by the ring precipitin method (Bancroft *et al.*, 1960). Serological controls included saline vs. antiserum, virus vs. normal serum, and healthy cowpea sap vs. antiserum when sap from infected plants was used in the tests.

RESULTS

Symptomatology

In contrast to shock and partial recovery symptoms of some plant virus diseases, chlorotic mottle symptoms of CCMV were produced on each new leaf throughout the life of the plant. Although the degree of yellowing sometimes varied from leaf to leaf, the yellowing did not diminish later or affect the color of new developing leaves.

Time Course Assays Measuring Specific Infectivity and Virus Growth

The specific infectivity and nucleoprotein concentration of CCMV were measured by harvesting representative samples from infected plants at different times after inoculation. The same relative infectivities and concentrations were obtained whether the seeding date was staggered and harvests were made at the same time or there was one

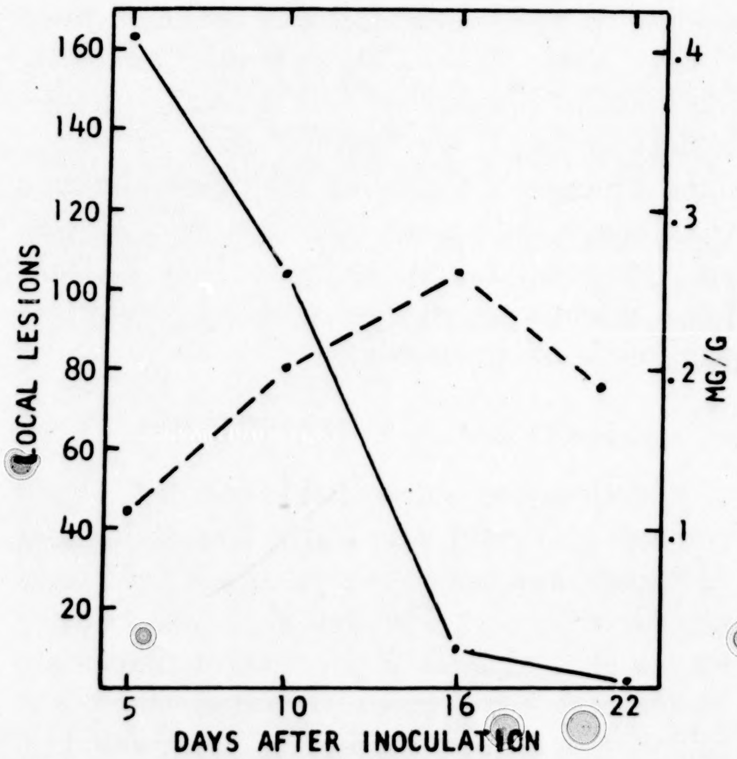

FIG. 2. Virus nucleoprotein concentration (broken line, milligrams of virus per gram of plant tissue) and specific infectivity (solid line) of CCMV grown in inoculated, primary cowpea leaves.

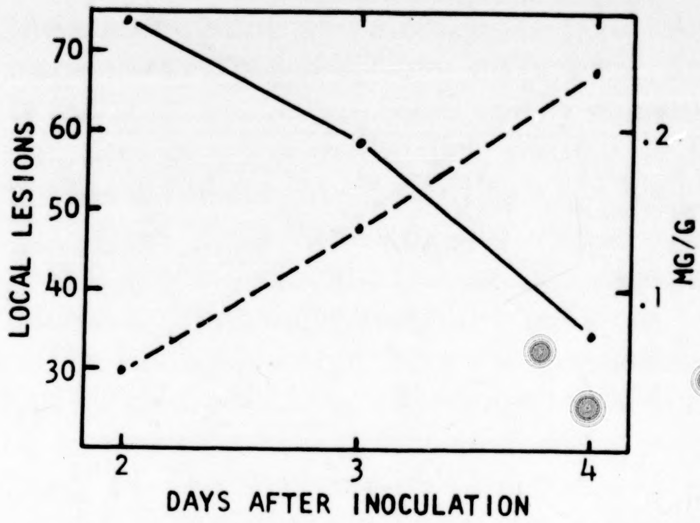

FIG. 3. Virus nucleoprotein concentration (broken line, milligrams of virus per gram of plant tissue) and specific infectivity (solid line) of CCMV grown in inoculated, primary cowpea leaves.

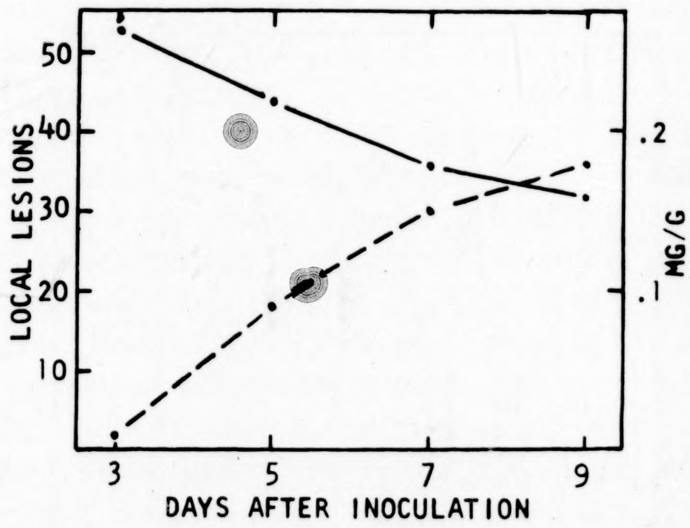

FIG. 4. Virus nucleoprotein concentration (broken line, milligrams of virus per gram of plant tissue) and specific infectivity (solid line) of CCMV grown in inoculated, primary cowpea leaves.

inoculation date and harvests were staggered and frozen until termination of the experiment. Typical curves representing amount of virus nucleoprotein, sap infectivity, and specific infectivity of virus from whole plants are illustrated in Fig. 1. In 20–30 days, specific infectivity dropped to 5–10% of an initial harvest at about 5 days. In time-course assays with inoculated primary leaves only specific infectivity declined from the value at an early harvest at 2 days after inoculation to those at all subsequent harvests (Figs. 2–4). Active virus could be obtained 1 day after inoculation, and it usually had a lower specific infectivity level than 2- and 3-day virus preparations. However, 1-day virus optical ratios at 240:260 mμ and 280:260 mμ were sufficiently dissimilar from later harvests to lead me to suspect that the 1-day preparation had different biophysical properties. Dissimilarities could be due to immature and incomplete virus components or plant impurities that were relatively more important in minute quantities of virus.

Virus from Leaves of Different Ages

To determine the effect of leaf age on virus specific infectivity and concentration, plants infected 50 days were divided into 3 parts. Although virus nucleoprotein concentration was similar in leaves of different ages, specific infectivity was highest in the youngest leaves that had been infected the shortest period (experiment 1, Table 1). When a similar length of infection period was involved, the specific infectivity level was similar for the primary and first 2 leaves and only slightly lower for virus from the newest growth on plants 65 days old (experiment 2, Table 1). When plants of different ages (10 and 30 days) were inoculated with the same inoculum at the same time, CCMV multiplied more rapidly in the younger plants; specific infectivity, however, was similar with plants of both ages.

Effect of Temperature

Plants infected 7 days with CCMV were transferred to two growth chambers with temperatures of 16° and 32° and a 12-hour photoperiod. Two harvests, in addition to the initial 7-day control, indicated that specific infectivity increased at 16° and decreased at 32° (Fig. 5). The virus nucleoprotein concentration increased at 16° and remained about constant at 32° (Fig. 5).

Effect of Antioxidants

Normal plant constituents of an oxidative nature that could be inhibited by sodium diethyldithiocarbamate (0.01 *M*), sodium sulfite (0.1%), ascorbic acid (0.1 *M*), or thioglycolic acid (0.1%) were not responsible for differences in specific infectivity. When leaf tissue was extracted in buffer plus each of the four chemicals, the infectivity decreased similarly for virus from both young and old infections.

TABLE 1

A Comparison of Concentration and Specific Infectivity of CCMV Extracted and Purified from Leaves of Different Ages

Expt. no.	Leaves harvested	Plant age, days from seeding	Length of infection in leaves (days)	Amount of purified virus (mg/g)[a]	Average number of lesions/half leaf	
					Sap	Equalized[b]
1	Primary leaves	58	50	0.22	7	20
	First 2 leaves	58	35–40	0.29	27	58
	Upper leaves	58	15–30	0.27	68	66
2	Primary leaves	15	7	0.04	—[c]	84
	First 2 leaves	25	7–10	0.80	—	80
	Tip leaves	65	7–10	0.25	—	65

[a] Milligrams of virus per gram of plant tissue.
[b] Specific infectivity based on optical equalization at 260 mμ.
[c] Lesion numbers were too high for a satisfactory assay.

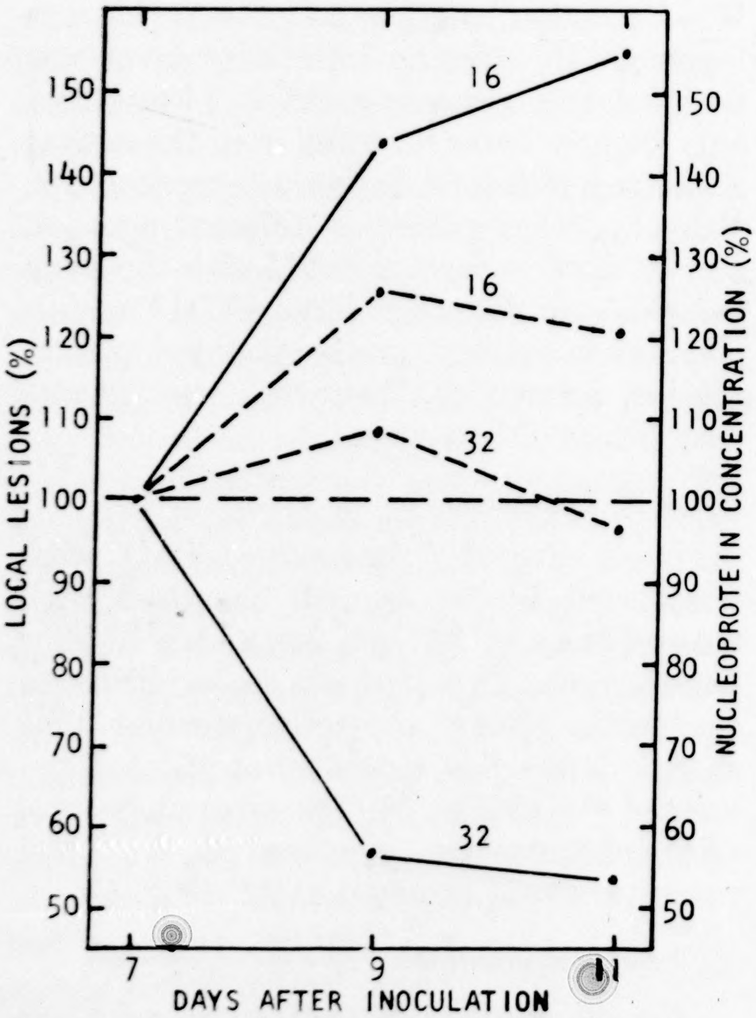

Fig. 5. Effect of 16° and 32° temperatures on virus nucleoprotein concentration (broken lines, milligrams of virus per gram of plant tissue) and specific infectivity (solid lines) of CCMV grown in inoculated, primary cowpea leaves. 100% nucleoprotein = 0.10 mg virus per gram of leaf tissue. 100% local lesions = 31 and 25 lesions per half-leaf for 16° and 32°, respectively.

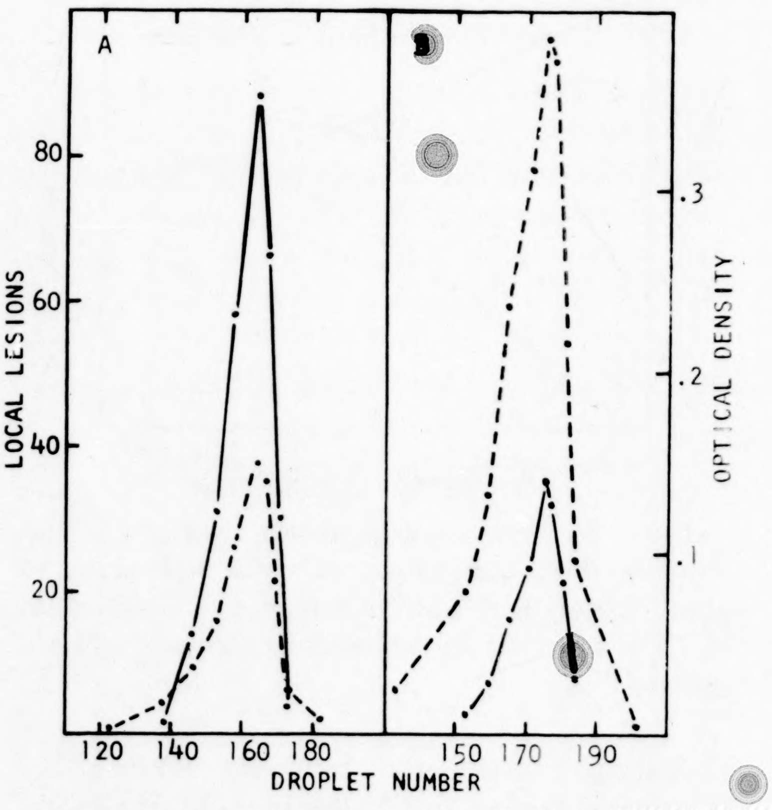

Fig. 6. A comparison of infectivity (solid lines) and optical density (broken lines) of CCMV obtained by the droplet method from centrifuged sucrose columns. A. Virus from 7-day infection. B. Virus from 35-day infection.

Effect of Purification

Changes, caused by manipulation and purification procedures, in the infective nature of CCMV from infections of different ages were checked by several methods. The rate of inactivation was similar for virus from young and old infections at 5° and −29°,

the manipulating and storage temperatures, respectively. Aggregation differences between treatments were ruled out by comparing sap extracted in distilled water and in buffers of different molarities. Moreover, low speed centrifugation, (10 minutes at 20,000 *g*), caused slight but similar losses in infectivity for young and old infections.

The nonrelationship between purification and specific infectivity was supported further by serological tests of unpurified sap. Tissue from 14- and 42-day-old infections was ground in phosphate buffer and centrifuged 10 minutes at 20,000 *g*. At sap dilutions of 1:20 and 1:100, the 14-day infection produced 195 and 89 local lesions per half-leaf, while none was produced by corresponding preparations from the 42-day treatment; virus activity was detectable in the 42-day-old infection, but not at those dilutions. Both sap extracts, however, reacted in the ring precipitin test to a dilution of 1:2560 after 2 hours at room temperature.

The most critical specific infectivity tests were made after the purification step of rate or equilibrium density gradient centrifugation (Brakke, 1960). Droplets collected from the bottom of sucrose columns and measured optically at 260 mμ revealed a single virus zone, in the same relative position, from both young and old infections. Furthermore, the zones were directly related to infectivity (Fig. 6). Figure 6 shows the physical similarity between virus from young and old infections and graphically illustrates the sharp contrast in specific infectivity.

DISCUSSION

A decline in specific infectivity with increasing age of infection has been demonstrated for at least 5 plant viruses: (1) tomato bushy stunt virus (TBSV) (Kassanis, 1952), (2) turnip yellow mosaic virus (TYMV) (Matthews and Lyttleton, 1959), (3) alfalfa mosaic virus (AMV) (Kuhn and Bancroft, 1961), (4) broad bean mottle virus (BBMV) (Kodama and Bancroft, 1964), and (5) CCMV. To demonstrate the decline with TBSV and TYMV, it was necessary to move infected plants to relatively high temperatures of 33–35°. For AMV, BBMV, and CCMV *in vivo* inactivation occurred at greenhouse temperatures. However, the inactivation rate was increased for AMV and CCMV (Fig. 5) at elevated temperatures. While AMV specific infectivity declined slightly at 16° (Kuhn, 1960), both specific infectivity and virus nucleoprotein concentration of CCMV increased. Presumably, increase of CCMV specific infectivity at 16° was due to virus multiplication coupled with a slow rate of inactivation or none.

As with TYMV (Matthews and Lyttleton, 1959) and AMV (Kuhn and Bancroft, 1961), no differences in biophysical or serological properties were detected in CCMV preparations with differing specific infectivities. Thus, subtle differences must be sought to explain the biological change. The relatively high specific infectivity in new systemically infected leaves (Table 1) suggests that the infectious quality of CCMV is not genetically deteriorating during the course of an infection. Hypothetically, particles of inferior infectivity might be formed and lead to new particles. If this were correct, virus concentration and specific infectivity would decrease in new developing leaves. Because of the importance of intact virus ribonucleic acid (RNA) (Gierer, 1958; Haselkorn, 1962), demonstration of cleaved RNA within particles of BBMV (Kodama and Bancroft, 1964) offers the best explanation of specific infectivity change to date. The generality and cause of RNA scission should be considered in determining the mechanism of *in vivo* virus inactivation.

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VIROLOGY 25, 15–21 (1965)

The Fine Structure of Gladiolus Virus

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Electron micrographs of *Gladiolus* virus (GV) preparations negatively stained with neutral phosphotungstic acid indicate that the GV capsid, 29 mμ in diameter, is icosahedral with 42 capsomeres. This conclusion is further supported by use of a rotation technique for photographic enhancement of detail.

INTRODUCTION

Recently a new virus has been isolated from a *Gladiolus* hybrid ("spic and span") in South Australia which shows some similarities to certain isolates of cucumber mosaic virus (CMV). This *Gladiolus* virus (GV) produced mosaic symptoms similar to those of CMV on cucumber (*Cucumis sativus* L. var. Davis Perfect). It also reacted similarly to CMV on *Chenopodium amaranticolor* Coste et Reyn. but did not react on hosts which are usually regarded as good diagnostic plants for CMV (Smith, 1957). For example, GV failed to produce local lesions on cowpea (*Vigna sinensis* Endl. var. Black Eye) but caused systemic infections showing a faint mottle. GV infected *Nicotiana glutinosa* L. but failed to induce visible symptoms and produced well defined ring spots on some hosts such as *Antirrhinum* sp. and *Datura stramonium* L. when grown under low light conditions. The vector for GV is not known. It is, however, seed transmitted in *N. glutinosa* and in soybean, *Glycine max* (L) Merr. var Lincoln. Detailed studies of GV are still in progress, but they have already shown that it is distinct from CMV; this work will be reported in a later communication. We have studied the fine structure of GV by examining negatively stained preparations and have found that the capsid is an icosahedron about 29 mμ in diameter with 42 capsomeres. The terminology proposed by Lwoff *et al.* (1962) has been used throughout this paper.

MATERIALS AND METHODS

Partial purification of virus. The virus was cultured in cucumber seedlings (*Cucumis sativus* L. var. Davis Perfect). Cucumber plants were inoculated when at the cotyledonary stage and harvested 10–14 days later when the leaves showed prominent symptoms. The purification procedure described by Scott (1963) for CMV was used; it yielded 50–300 mg virus per kilogram of leaf material.

Electron microscopy. Purified virus was mixed with an equal volume of freshly prepared 2% PTA (phosphotungstic acid neutralized with KOH to pH 7). Drops of this mixture were immediately placed on copper grids which had been coated with collodion and backed with carbon. The excess liquid was removed with lens tissue. The grids were air dried at room temperature and examined in the Siemens Elmiskop I

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Suppression of Virus Infectivity in Diseased Plant Tissue Following Treatment with an Antiviral Factor from Virus-Infected Plants

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An antiviral factor (AVF), which is produced in virus infected plants only, was applied in purified form to leaf tissues previously infected with a plant virus. The AVF suppressed markedly the infective virus content within the treated leaves, thus providing evidence for its *in vivo* action. The properties of the AVF as described in the present report and in former ones led to conclusions concerning its possible site of action, its host and virus specificity, and its similarity to and difference from interferon.

INTRODUCTION

An antiviral factor (AVF), which occurs in virus-infected plants and not in noninfected plants, was described in 1962 (Sela and Applebaum, 1962) and later was separated by column chromatography (Sela *et al.*, 1964). The purified, highly active material, designated "AVF preparation," consisted of protein and RNA, but it has not yet been finally defined.

In previous experiments, infective inocula mixed *in vitro* with AVF lost most of their infectivity. This could be attributed to a direct effect of the AVF on the virus or on host cells. The study reported herein comprises an attempt to test AVF activity *in vivo* by examining its effect on virus-infected leaves.

EXPERIMENTAL

The "AVF preparations" used throughout the following series of experiments were prepared from leaves of *Nicotiana glutinosa* L. infected with tobacco mosaic virus (TMV) (Sela *et al.*, 1964). The AVF preparations were dissolved in 0.01 *M* sodium phosphate buffer of pH 7.6 (the standard buffer) and their concentration was adjusted to an absorbancy of 0.090–0.150 units at 260 mμ in a Beckman DU spectrophotometer. The respective protein concentrations of these solutions, as determined with the phenol reagent (Lowry *et al.*, 1951), were in the range of 6–10 μg protein per milliliter.

AVF activity was tested on young leaves of different plants inoculated with TMV 10 months prior to AVF treatment, or with one of two strains of cucumber mosaic virus (CMV) 2 months prior to AVF treatment. One of these CMV isolates does not produce local lesions on *Zinnia elegans* Jacq., whereas the other one provokes local lesions and also spreads systemically in that plant (the latter strain is thus designated as CMV-LL). The infected leaves were harvested and their midribs removed. One of the two symmetrical half-leaves thus obtained was immersed in the AVF solution (treated half-leaf) whereas the second was immersed for the same length of time in the standard buffer (control half-leaf). After 24–48 hours the half-leaves were taken out of their respective solutions and washed thoroughly with the standard buffer. Each leaf was homogenized in a certain volume of the standard buffer and the sap obtained was used as inoculum.

Virus infectivity of the saps from treated and control half-leaves was determined by the half-leaf test (Samuel and Bald, 1933). TMV-containing saps were applied to *Datura stramonium* L. half-leaves; and

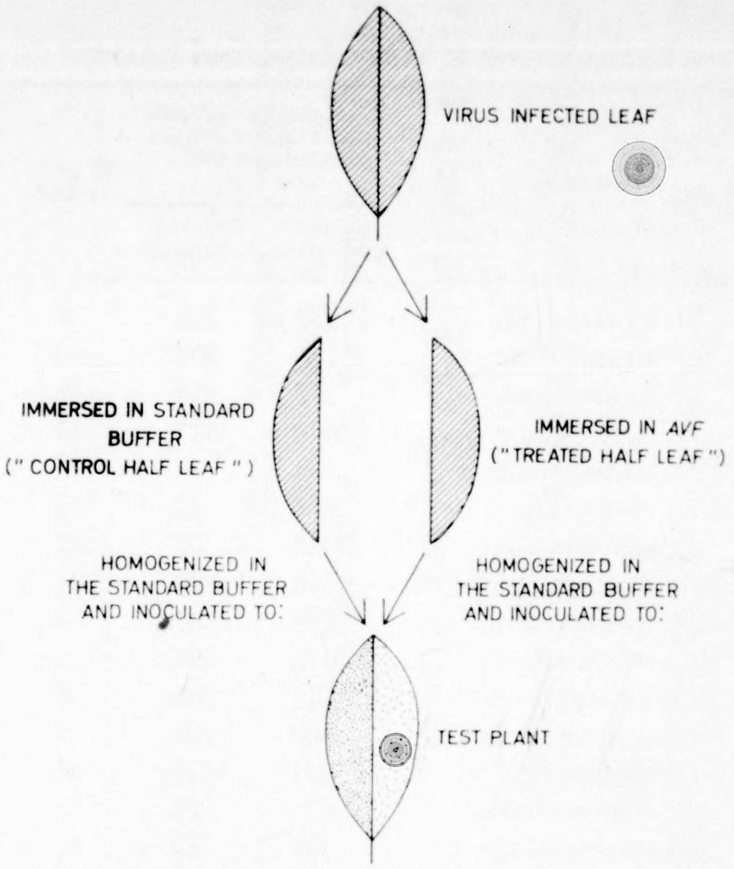

FIG. 1. Schematic illustration of the procedure employed to test AVF activity on virus-infected leaves.

CMV-containing saps, *Chenopodium amaranticolor* Coste & Reyn. half-leaves.

Virus inocula were diluted to such a concentration that the number of local lesions produced was still on the linear part of the lesion-concentration curve.

At least 6 leaves of *D. stramonium* or 12 leaves of *C. amaranticolor* were used for each experiment. Decrease in virus infectivity was calculated as percentage protection on the basis of 0% protection on the control half-leaves (Nitzany and Cohen, 1960).

When treating infected *Z. elegans* or *Gomphrena globosa* L. leaves with AVF, the same procedure was employed, but with opposite leaves instead of half-leaves. The above-described procedure is schematically summarized in Fig. 1.

RESULTS

In all our tests (44 tests with TMV and 13 tests with CMV), considerable decreases in virus infectivity were recorded in the treated half-leaves, as compared to the corresponding control half-leaves. This is demonstrated in Table 1, where examples of low, medium, and high antiviral potencies are presented.

Special control experiments were designed to rule out the possibility that some effective AVF still remained on or within the AVF-treated leaves in spite of the washing. If not all the effective AVF had been washed out, the residual AVF would have caused a decrease in virus infectivity when mixed in the test tube with the virus in homogenization. In these special control experiments, dilute inocula of infective viruses were added to the saps obtained from noninfected leaves treated with AVF or with buffer. No decreases in infectivity were observed (see Table 1, "noninfected leaves"), which shows that no effective AVF remained on or in the leaves after they have been washed.

DISCUSSION

The data in the present report clearly demonstrate the *in vivo* antiviral effect of the AVF on infected tissues. The AVF suppresses markedly the infective virus content of leaf-tissues that have been infected with virus for a long time prior to the AVF treatment. These results provide evidence that one site of action of the AVF is inside the infected tissue. This may be either a direct effect on the virus particles, or an indirect effect on virus production within the infected tissue produced by interfering with some phase of the metabolic processes leading to the synthesis of new mature virus particles. Unless there is more than one mode of action of the AVF, the above results preclude the possibility of any initial stage of virus infection, such as attachment to host cells, penetration, neutralization of host acceptors, as the possible site of action of the AVF.

The present data provide some new evidence concerning virus and host specificity of the action of the AVF. These data are in agreement with earlier information (Sela and Applebaum, 1962), namely, that no virus or host specificity could be shown in regard to the action of the AVF. The AVF prepared from TMV-infected *N. glutinosa* leaves is active against TMV as well as against two strains of CMV and

TABLE 1

EFFECT OF ANTIVIRAL FACTOR ON INFECTIVITY OF EXTRACTS FROM VIRUS-DISEASED LEAVES[a]

Virus	Host plant	Virus concentration (grams leaves/ml standard buffer)	Test plant	Total number of local lesions on half-leaves inoculated with saps from: Control half-leaves	Treated half-leaves	% protection
Noninfected	Tobacco	1:150[b]	*D. stramonium*	253	208	0
Noninfected	Tobacco	1:150[b]	*D. stramonium*	179	200	0
Noninfected	Tobacco	1:150[b]	*D. stramonium*	175	215	0
TMV	Tobacco	1:40	*D. stramonium*	1548	847	45
TMV	Tobacco	1:120	*D. stramonium*	131	33	75
TMV	Tobacco	1:150	*D. stramonium*	1685	42	97
TMV	*Z. elegans*	1:4	*D. stramonium*	2043	52	97
TMV	*G. globosa*	1:8	*D. stramonium*	595	394	34
Noninfected	Cucumber	1:150[b]	*D. stramonium*	278	285	0
Noninfected	Cucumber	1:150[b]	*D. stramonium*	432	406	0
Noninfected	Cucumber	1:6[c]	*C. amaranticolor*	171	168	0
Noninfected	Cucumber	1:6[c]	*C. amaranticolor*	109	108	0
CMV	Cucumber	1:6	*C. amaranticolor*	241	125	48
CMV	Cucumber	1:6	*C. amaranticolor*	244	79	68
CMV	Cucumber	1:6	*C. amaranticolor*	284	50	82
CMV-LL	Cucumber	1:6	*C. amaranticolor*	139	49	64
CMV-LL	Cucumber	1:6	*C. amaranticolor*	49	1	98
CMV-LL	*Z. elegans*	1:3	*C. amaranticolor*	167	9	95

[a] All tests were analyzed by the t test for related samples ($\alpha = 0.01$).

[b] Inoculum of TMV was diluted with noninfected leaf extracts (the latter previously diluted to the same dilutions as the comparable extracts of infected leaves) to attain the 1:150 final TMV concentration.

[c] Inoculum of CMV was diluted in the same manner as the TMV inoculum so as to give the 1:6 final CMV concentration.

two strains of potato virus Y. This AVF is very active against viruses in a wide range of host plants, which belong to different botanical families, such as tobacco (Solanaceae), *Z. elegans* (Compositae), *G. globosa* (Amarantaceae), and cucumber (Cucurbitaceae).

The AVF seems to resemble interferon in several biological properties. Both materials have antiviral activity, they are produced only in virus-infected tissues, they easily diffuse from infected cells to noninfected tissues (Sela and Applebaum, 1962), and they have no virus specificity. However AVF differs from interferon in the fact that the latter is host specific (Isaacs, 1963) whereas AVF is not.

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The use of antiviral chemicals to protect plants
against some viruses transmitted by aphids.
Virology, 25 (1):83-87, 4 tables, 1965.
Microfilm no.

...als to Protect Plants against Some ...smitted by Aphids[1]

...Jr.,[2] and R. K. Chapman[3]

...sity of Wisconsin, Madison, Wisconsin

...l October 7, 1964

...nst aphid-transmitted plant viruses in green-
...er such chemicals act differently against aphid
...s. Sprays of Cytovirin (metabolite of *Strep-*
... aphid transmission of potato virus Y (PVY)
... those viruses were mechanically transmitted.
...-transmitted pea virus 2 and cabbage virus B.
...ffectiveness of Cytovirin. Cytovirin applied
...re with aphid acquisition of PVY and had no
direct effect on PVY when fed to viruliferous aphids.

Trichothecin (metabolite of *Trichothecium roseum* Link) reduced PVY infections of tobacco by about one-half when the virus was aphid-transmitted but had no effect on cabbage virus B.

Milk failed to prevent aphids from transmitting PVY to tobacco plants.

INTRODUCTION

As most plant viruses are transmitted in nature by insects, practical antiviral chemicals must have the ability to prevent multiplication of insect transmitted viruses within the plant hosts. When applied to plant foliage, many chemicals will prevent virus infection of plants from mechanical inoculation (Matthews and Smith, 1955). The same chemicals are often less effective against viruses transmitted by insects (Bradley and Ganong, 1957; Simons, 1960).

This paper presents the results of greenhouse experiments with antiviral chemicals, reported to prevent infections from mechanically transmitted viruses, against some viruses transmitted by aphids. The materials tested were Cytovirin,[4] a metabolite of *Streptomyces* sp. (Gray, 1957); Trichothecin,[4] produced by *Tricothecium roseum* Link (Gupta and Price, 1952); and milk (Hare and Lucas, 1959).

MATERIALS AND METHODS

General. Tobacco seedlings in the 3–4 leaf stage, cauliflower seedlings with two true leaves, and pea seedlings with 2–3 leaves were used as test plants, depending on the virus to be tested. Tobacco plants which were to be used as indicators for potato virus Y (PVY) were mechanically inoculated with potato virus X (PVX) 2 days before use as test plants.

The cabbage aphid, *Brevicoryne brassicae* (Linnaeus) was used as the vector for cabbage virus B (CVB) and the green peach aphid, *Myzus persicae* (Sulzer), for all other

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[4] The authors are grateful to Dr. R. A. Gray, Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey, for the gifts of Cytovirin and to Dr. G. G. Freeman, Imperial Chemicals Industries, Stevenston, Ayrshire, Scotland for the Trichothecin.

TABLE 1

EFFECT OF ANTIVIRAL FACTOR ON INFECTIVITY OF EXTRACTS FROM VIRUS-DISEASED LEAVES[a]

Virus	Host plant	Virus concentration (grams leaves/ml standard buffer)	Test plant	Total number of local lesions on half-leaves inoculated with saps from:		% protection
				Control half-leaves	Treated half-leaves	
Noninfected	Tobacco	1:150[b]	*D. stramonium*	253	208	0
Noninfected	Tobacco	1:150[b]	*D. stramonium*	179	200	0
Noninfected	Tobacco	1:150[b]	*D. stramonium*	175	215	0
TMV	Tobacco	1:40	*D. stramonium*	1548	847	45
TMV	Tobacco	1:120	*D. stramonium*	131	33	75
TMV	Tobacco	1:150	*D. stramonium*	1685	42	97
TMV	*Z. elegans*	1:4	*D. stramonium*	2043	52	97
TMV	*G. globosa*	1:8	*D. stramonium*	595	394	34
Noninfected	Cucumber	1:150[b]	*D. stramonium*	278	285	0
Noninfected	Cucumber	1:150[b]	*D. stramonium*	432	406	0
Noninfected	Cucumber	1:6[c]	*C. amaranticolor*	171	168	0
Noninfected	Cucumber	1:6[c]	*C. amaranticolor*	109	108	0
CMV	Cucumber	1:6	*C. amaranticolor*	241	125	48
CMV	Cucumber	1:6	*C. amaranticolor*	244	79	68
CMV	Cucumber	1:6	*C. amaranticolor*	284	50	82
CMV-LL	Cucumber	1:6	*C. amaranticolor*	139	49	64
CMV-LL	Cucumber	1:6	*C. amaranticolor*	49	1	98
CMV-LL	*Z. elegans*	1:3	*C. amaranticolor*	167	9	95

[a] All tests were analyzed by the t test for related samples ($\alpha = 0.01$).

[b] Inoculum of TMV was diluted with noninfected leaf extracts (the latter previously diluted to the same dilutions as the comparable extracts of infected leaves) to attain the 1:150 final TMV concentration.

[c] Inoculum of CMV was diluted in the same manner as the TMV inoculum so as to give the 1:6 final CMV concentration.

two strains of potato virus Y. This AVF is very active against viruses in a wide range of host plants, which belong to different botanical families, such as tobacco (Solanaceae), *Z. elegans* (Compositae), *G. globosa* (Amarantaceae), and cucumber (Cucurbitaceae).

The AVF seems to resemble interferon in several biological properties. Both materials have antiviral activity, they are produced only in virus-infected tissues, they easily diffuse from infected cells to noninfected tissues (Sela and Applebaum, 1962), and they have no virus specificity. However AVF differs from interferon in the fact that the latter is host specific (Isaacs, 1963) whereas AVF is not.

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The Use of Antiviral Chemicals to Protect Plants against Some Viruses Transmitted by Aphids[1]

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Antiviral chemicals were tested against aphid-transmitted plant viruses in greenhouse experiments to determine whether such chemicals act differently against aphid and mechanical transmission of viruses. Sprays of Cytovirin (metabolite of *Streptomyces* sp.) were less effective against aphid transmission of potato virus Y (PVY) and cucumber mosaic virus than when those viruses were mechanically transmitted. Cytovirin was ineffective against aphid-transmitted pea virus 2 and cabbage virus B. A wetting agent did not increase the effectiveness of Cytovirin. Cytovirin applied to PVY-infected foliage did not interfere with aphid acquisition of PVY and had no direct effect on PVY when fed to viruliferous aphids.

Trichothecin (metabolite of *Trichothecium roseum* Link) reduced PVY infections of tobacco by about one-half when the virus was aphid-transmitted but had no effect on cabbage virus B.

Milk failed to prevent aphids from transmitting PVY to tobacco plants.

INTRODUCTION

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This paper presents the results of greenhouse experiments with antiviral chemicals, reported to prevent infections from mechanically transmitted viruses, against some viruses transmitted by aphids. The materials tested were Cytovirin,[4] a metabolite of *Streptomyces* sp. (Gray, 1957); Trichothecin,[4] produced by *Tricothecium roseum* Link (Gupta and Price, 1952); and milk (Hare and Lucas, 1959).

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General. Tobacco seedlings in the 3–4 leaf stage, cauliflower seedlings with two true leaves, and pea seedlings with 2–3 leaves were used as test plants, depending on the virus to be tested. Tobacco plants which were to be used as indicators for potato virus Y (PVY) were mechanically inoculated with potato virus X (PVX) 2 days before use as test plants.

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viruses. Apterous aphids were used throughout and were starved 2–4 hours before being given 5 minutes' access to an infected plant.

Tests with Cytovirin. Cytovirin was tested against aphid-transmitted PVY in Havana 38 and Havana 425 tobacco, cucumber mosaic virus (CMV) in Havana 38 tobacco, pea virus 2 (PV2) in Alaska peas, and CVB in Dry Weather cauliflower. It was sprayed onto the plants in a water solution to which a wetting agent, Triton B-1956 (modified phthalic glycerol alkyd resin), was added for the peas and cauliflower. Two hours later, aphids were transferred in groups of two from infected plants to test plants, where they remained 18–24 hours.

For comparison, Cytovirin was also tested against mechanically inoculated PVY and CMV in Havana 38 tobacco. Tobacco plants were sprayed with Cytovirin. Two hours later they were inoculated by lightly dusting a leaf on each test plant with Carborundum powder and then rubbing sap, expressed from an infected plant, onto the leaves with a glass spatula.

An effort to obtain greater reduction of PVY infections was made in two ways. In one test, Triton B-1956 was added to the solution of Cytovirin (1 drop per 40 ml) to increase coverage of the leaf surface. In another test, additional sprays were applied to the plants 24 hours before and after inoculation by aphids.

The effect of Cytovirin on the acquisition of PVY was determined by spraying tobacco plants, which had 6–8 leaves and which had been infected with PVY for 10–14 days, with Cytovirin (25 ppm) and then assaying them at intervals with aphid acquisition tests. Green peach aphids were placed on infected plants for 2 minutes and then transferred singly to indicator plants. Half of the infected plants were then sprayed with Cytovirin, and the assay procedure with aphids was repeated 1, 2, 4, and 6 hours after spraying. The aphid remained on the indicator plants for at least 8 hours, and the plants were observed for 2 weeks for PVY symptoms.

To determine whether Cytovirin had any direct effect on PVY, green peach aphids were fed on a PVY-infected tobacco plant and then on a solution of Cytovirin (100 ppm). The solution was placed in the hollow plug-type cap of a 1-inch vial. A piece of animal membrane (Baudruche) was placed across the open end of the cap; the cap was inserted into the vial, which had the lower half removed. After they had made two short feeding probes in a PVY-infected tobacco leaf, the aphids were placed on the membrane to probe for a total of 1 minute. The feeding material was 5% sucrose to which the Cytovirin was added. Chalfant and Chapman (1962) inactivated CVB on aphids' stylets by allowing them to feed through a membrane on 1.25% formaldehyde, so this chemical and concentration in 5% sucrose was used as a check to determine whether the stylets penetrated the membrane. A check of 5% sucrose was used also. After feeding on a solution, each aphid was placed on a healthy tobacco seedling which was observed for PVY symptoms. A total of 20 aphids was tested on each solution in two tests of ten each.

Tests with Trichothecin. Trichothecin was tested against PVY in Havana 38, Havana 425, and White Burley tobacco and against CVB in Dry Weather cauliflower. The methods used were the same as previously described under Tests with Cytovirin.

Test with milk. Havana 38 tobacco plants were dipped in undiluted homogenized whole milk and allowed to dry for 1 hour. One green peach aphid, previously fed on a PVY-infected plant, was then placed on each test plant for several hours.

RESULTS AND DISCUSSION

Tests with Cytovirin

Results. The results with PVY and CMV were similar to those obtained by Simons (1960), who found that Cytovirin reduced the susceptibility of *Capsicum annuum* Linnaeus more to PVY transmitted by rubbing than when the virus was transmitted by aphid feeding. In our tests, Cytovirin at 25 ppm significantly reduced the number of infections from aphid-transmitted PVY (Table 1), but even at 100 ppm it had no significant effect on CMV transmitted by aphids (Table 2). Cytovirin was more effective against the viruses when they were transmitted mechanically than when

transmitted by aphids. Five parts per million gave 79% reduction of PVY infections from mechanical inoculation whereas 25 ppm gave only 42% reduction in the same tobacco variety when the virus was aphid-transmitted (Table 1). With CMV, 5 ppm reduced infections from mechanical transmissions 51% while 100 ppm gave only a 7% reduction from aphid transmissions (Table 2).

There was little phytotoxicity to tobacco with concentrations of Cytovirin up to 10 ppm. Unfortunately, 10 ppm was not effective against PVY. Twenty-five parts per million produced necrosis on the leaves, and 50 and 100 ppm sometimes also caused stunting.

When additional sprays of Cytovirin (25 ppm) were applied 24 hours before and after inoculation of tobacco with PVY by aphids, 8 of 60 tobacco plants that received additional applications of Cytovirin and 19 of 60 untreated plants became infected by PVY, a reduction of 58%. Two additional sprays of Cytovirin on the plants 24 hours before and 24 hours after inoculation were no more effective than one spray 1 or 2 hours before inoculation.

PVY symptoms developed on 18, 17, and 22 plants (out of 40 each) sprayed, respectively, with Cytovirin (25 ppm) plus Triton B-1956 spreader, Cytovirin only, or spreader only. Apparently the spreader did not increase the effectiveness of Cytovirin.

When tobacco plants infected with PVY were sprayed with Cytovirin and aphids were allowed to feed on them before transference to indicator plants, there was a reduction in aphid-acquisition of PVY from both treated and untreated infected plants as the experiment proceeded (Table 3). The maximum reduction in efficiency of virus acquisition for the treated and untreated plants was 25 and 29%, respectively. This occurred at approximately the same time of day and probably was due to changes in aphid feeding behavior.

When aphids were fed Cytovirin through

TABLE 1

INFECTION OF TOBACCO PLANTS WITH PVY WHEN SPRAYED WITH CYTOVIRIN BEFORE INOCULATION

Tobacco variety	Conc. of Cytovirin (ppm)[a]	Number of plants per treatment	% infected by PVY		% reduction of PVY infections
			Cytovirin	Water	
Green peach aphid inoculation					
Havana 38	10	31	48	55	13
Havana 38	25	55	40	69	42[b]
Havana 38	50	40	55	73	25
Havana 425	25	40	23	63	64[b]
Havana 425	50	40	10	63	84[b]
Mechanical inoculation					
Havana 38	5	20	20	95	79[b]

[a] Slight phytotoxicity with 25 ppm, extremely phytotoxic with 50 ppm.

[b] Significant at 1% level (Chi square test).

TABLE 2

INFECTION OF HAVANA 38 TOBACCO PLANTS WITH CMV WHEN SPRAYED WITH CYTOVIRIN BEFORE INOCULATION

Conc. of Cytovirin (ppm)[a]	Number plants per treatment	% plants infected		% reduction of infections
		Cytovirin	Water	
Green peach aphid inoculation				
10	40	25	15	0
25	30	40	43	7[b]
100	30	40	43	7[b]
Mechanical inoculation				
5	29	48	97	51[c]

[a] Slight phytotoxicity with 25 ppm; extremely phytotoxic with 50 or 100 ppm.

[b] Not significant (Chi square test).

[c] Significant at 1% level (Chi square test).

TABLE 3

TRANSMISSION OF PVY FROM INFECTED TO HEALTHY TOBACCO PLANTS BY GREEN PEACH APHIDS BEFORE AND AT INTERVALS AFTER THE INFECTED PLANTS WERE SPRAYED WITH CYTOVIRIN (25 ppm)

Treatment[a]	% transmission before treatment	% reduction of transmissions at stated intervals after treatment			
		1 hr.	2 hr.	4 hr.	6 hr.
Cytovirin	49	18	24	14	22
Unsprayed	80	9	29	29	25

[a] 20 aphids per infected plant per time interval for 3 replications and 10 aphids per infected plant per time interval for 3 replications.

a membrane following acquisition of PVY, eight aphids fed on Cytovirin plus sucrose, two on formaldehyde, and eight on sucrose only transmitted PVY to tobacco plants. The fact that only two infections resulted from virus transmitted by aphids fed on formaldehyde indicated most of the stylets penetrated through the membranes.

PV2 symptoms developed in 10 of 18 pea plants sprayed with Cytovirin (25 ppm) and 8 of 18 untreated plants. Of 40 cauliflower seedlings sprayed with Cytovirin (25 ppm) and 40 untreated, 12 and 13, respectively, developed CVB symptoms. It was concluded that Cytovirin was ineffective against these viruses, at least in these host plants.

Discussion. PVY and CMV infections may have been reduced more in mechanically inoculated plants than in those inoculated by aphids because of better absorption of the chemical through the very small but visible wounds produced by rubbing the leaves. The rubbing action on the leaves may have carried the chemical inside them. Aphid stylets leave wounds invisible to the naked eye and there are very few compared with those made by rubbing. It is possible aphids may place a virus in the leaf so that it is protected from antiviral chemicals or the viruses may become more quickly established in plant tissue when transmitted by aphids.

The reduction of PVY infections was probably not related to mechanical loss of the inoculated tissue due to leaf necrosis, even though a large percentage of leaf area was often destroyed, because a high percentage of plants sprayed with 100 ppm developed CMV symptoms in spite of similar injury. The reduction in PVY transmission apparently was due to the effect of Cytovirin or its metabolites on the virus or host susceptibility.

The results from spraying PVY-infected plants prior to acquisition feeding by aphids indicates that Cytovirin does not immediately inactivate PVY in the host plant. Simons (1960) in similar tests, did not show a reduction of PVY titer until 2 days after treatment. The fact that as many aphids fed on Cytovirin solution through a membrane transmitted PVY as those receiving only sucrose also indicates that Cytovirin has no direct effect on PVY.

Tests with Trichothecin

Results. Ten parts per million of Trichothecin gave from 45 to 57% reduction of infections by aphid-transmitted PVY (Table 4). A concentration of 20 ppm failed to increase the amount of virus control. Five of 20 of both treated and untreated cauliflower plants developed CVB symptoms which indicated that Trichothecin had no effect on the transmission of CVB by aphids.

Discussion. Bradley and Ganong (1957) obtained almost complete prevention of PVY transmission with 3 ppm of Trichothecin. The failure to obtain results comparable to those of Bradley and Ganong (1957), in spite of higher rates used, may have been due to physiological differences in the plants caused by the method of rearing. As Trichothecin inhibits virus multiplication by changing the host plants' susceptibility (Stahman and Gothoskar, 1958), the effectiveness of the chemical must depend, in part, on the host physiology.

Test with Milk

Eighteen per cent of 40 tobacco plants treated with milk and 28% of the untreated plants developed PVY symptoms. The difference was not significant because 6 of the 11 infections in the untreated check occurred in one replication. Milk probably failed to prevent transmission of PVY by

TABLE 4
TRANSMISSION OF PVY BY THE GREEN PEACH APHID TO TOBACCO PLANTS SPRAYED WITH TRICHOTHECIN BEFORE INOCULATION

Tobacco variety	Conc. of Trichothecin (ppm)	Number of plants per treatment	% infected by PVY: Trichothecin	% infected by PVY: Water	% reduction of PVY infections
Havana 38	5	48	31	27	0
Havana 38	10	65	23	42	45[b]
Havana 425	10	30	23	53	57[b]
White Burley	10	39	18	39	54
Havana 38	20[a]	30	37	60	38
Havana 425	20	30	27	53	49

[a] Phytotoxic effects evident.
[b] Significant at 5% level (Chi square test).

aphids because it was unable to penetrate into the inoculated cells.

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Characteristics of Antigens and Antibodies Associated with the Prunus Necrotic Ringspot Virus Immune System[1]

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Two antigens, V and Q, are associated with Prunus necrotic ringspot infections of woody and herbaceous hosts. Only V (virus) is considered to be infectious, and the antigens are closely related as shown by agar immunodiffusion and absorption tests. Rabbits immunized with V produce two antibody types. One type reacts with both V and Q and the other reacts only with V. Antigen and antibody types are separable by electrophoresis in agar and by density gradient centrifugation. V can be degraded into Q at room temperatures by using high molarities of NaCl. Q particles were shown to have at least two types of antigenic determinants, and serological evidence indicates that Q particles from individual V particles may be partially heterogeneous. Particle size determinations for V (30 ± 10 mμ) and Q (4 ± 1 mμ) were made by a modified immunodiffusion test.

INTRODUCTION

Certain antigenic particles detected in virus-infected woody (*Prunus*) and herbaceous plant species and designated as V (virus) antigens by Willison *et al.* (1961) have been identified as the Prunus necrotic ringspot virus (NRSV) by Allen (1963, 1964). Frequently associated with the virus in these plant tissues were smaller antigenic particles designated Q. Preliminary serological investigations on the identity of the Q antigen indicated that the particles were not related to V. Results from sedimentation studies suggested that Q particles might be breakdown products of V or of another virus. The first possibility seemed unlikely, however, owing to the apparent lack of a serological relationship between V and Q. The second possibility also seemed unlikely because there was no biological or serological evidence suggesting the presence of another virus. A further suggestion was that Q might represent anomalous products of infection, yet the fact that Q was associated with V in species differing as widely as cherry and cucumber did not seem to support the hypothesis (Tremaine and Willison, 1962; Willison *et al.*, 1961).

Subsequent work (Tremaine *et al.*, 1963) indicated that the association between V and Q might be constant and that results to the contrary in the earlier work were due to variations in concentration of the antigens. Tests in this work involved serological indexing of cucumber plants inoculated from seeds of NRSV-infected sour cherry trees. Some cucumber plants apparently contained only V or Q, and others contained both antigens. After serial transfers in cucumber, however, both V and Q were detected in all isolates.

Continued investigation has identified the Q antigen as a naturally occurring subunit of V (Allen and Tremaine, 1963). The tests that led to these conclusions and that have partially characterized this complex antigen and antibody system are presented here.

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MATERIALS AND METHODS

Antigen and antibody. Isolates of NRSV from petals, seeds, or leaves of sour cherry trees were obtained from Ontario New York, and Wisconsin. Success in isolation of NRSV was determined serologically with specific antisera prepared by Fulton and Hamilton (1960) and Allen (1964). Antisera prepared at this laboratory, each of which reacted with both V and Q (Tremaine and Willison, 1962), were used to test for the association of the Q antigen with all NRSV isolates used in this work. Antiserum prepared by Fulton to the G isolate of NRSV was also used because it too reacted with both antigens. In all studies, assays for V or Q were made in immunodiffusion plates with an antiserum reactive with both antigens. The respective precipitin zones were identified by their relative positions or by their coalescence with V or Q zones formed by a standard V-Q preparation included in the assay plates. In several instances, the V antigen was identified with antiserum reactive with it alone.

Antigens were separated from cherry petals and cucumber cotyledons in a Waring blendor containing buffer (the ratio of buffer to tissue was 3:1, ml/g, with petals and 1.5:1 for cucumber) composed of 0.02 *M* Na_3PO_4 and 0.04 *M* sodium diethyldithiocarbamate and adjusted to pH 8.0 with thioglycolic acid (Hampton and Fulton, 1961). Petal extracts were clarified with chloroform (Allen, 1964), and those from cucumber cotyledons by titration to pH 5.0 with HCl. Chloroform or pH 5.0 treatments were followed by differential centrifugation (13,000 *g* for 20 minutes and 59,000 *g* for 5 hours); the pellets were suspended in 0.02 *M*, pH 8.0 Tris-HCl [tris(hydroxymethyl)aminomethane - hydrochloride] containing 0.015 *M* sodium azide, and the final solution was given a low speed centrifugation. These procedures generally extracted both V and Q from cucumber and only V from cherry petals, though another procedure extracted both antigens from petals (Willison *et al.*, 1961). Some serological tests on herbaceous plants were made with sap expressed from the tissue with pliers.

The concentration of antigen preparations is denoted by digits representing the number of grams (fresh weight) of starting material per milliliter of final preparation followed by the symbol ×. The serological dilution end point of these preparations was determined by diffusing twofold serial dilutions of the preparation against antiserum in immunodiffusion plates arranged as in Fig. 4. The greatest dilution, e.g., 1:8 or 1:16, to form a precipitate was the dilution end point.

All antisera except 25, 26, and 27 were produced in rabbits by a single intramuscular thigh injection of a virus preparation emulsified in Freund's complete adjuvant (Difco, Detroit, Michigan). Sera 25, 26, and 27 were produced by 5, 5, and 4 intramuscular injections with adjuvant, respectively. Antibody concentrations were determined by dilution end point titrations as with antigens. Antisera were freeze-dried immediately after harvest and reconstituted with distilled water (100 mg/ml) as needed.

Serology and electrophoresis. Immunodiffusion tests were made in plastic petri dishes containing a 3-mm layer of agar in which 6-mm reactant wells were cut with a cork borer leaving a distance of 6 mm between well peripheries. Agar [Ionagar no. 2, British Drug Houses (Canada) Ltd., Toronto, Ontario] was prepared at a concentration of 0.7% with 0.015 *M* sodium azide and 0.15 *M* NaCl.

Absorption tests were done either by adding 10-mg increments of dried antiserum to 1 ml of purified virus solution in glass tubes or by adding 0.1 ml increments of a purified virus preparation to 1 ml of antiserum. The tubes were stored at 24° for 3 hours, and immune precipitates were removed by low speed centrifugation. A fraction of the supernatant was tested in immunodiffusion plates after each increment of antiserum or antigen to determine loss or build up of antigen or antibody. In those cases where absorption did not occur, tests were repeated at 0° to enhance reactions between weakly reacting systems (Boyd, 1956).

Agar electrophoresis was carried out in Perspex trays that supported a horizontal

agar layer (165 × 57 × 6 mm) and vertical agar extensions (37 × 57 × 6 mm). The vertical extensions, which connected with the electrode buffer, were reinforced with filter paper strips. For electrophoresis of antigens, the agar consisted of 0.7% Ionagar in 0.02 *M*, pH 8.0 Tris-HCl buffer. For electrophoresis of antibodies, 0.02 *M* NaCl was added to the agar and to the electrode buffer that consisted of 0.02 *M*, pH 8.0 Tris-HCl. A variable voltage (500 DC volts) power supply was used. During electrophoresis, the agar was cooled either by running tap water that contacted the tray or by conducting the tests in a cold room.

To separate antibodies, antiserum was dialyzed overnight against electrophoresis buffer and placed in 3 × 10 mm wells, two of which were cut in the agar layer 30 mm from the anode end and perpendicular to the migration path. After filling the wells, a plastic film was placed on the agar to prevent drying. Serum was electrophoresed for 4 hours (6 volts/cm), and antibodies were located as reported by Brown (1958) by cutting 6-mm samples from the migration path with a cork borer (Fig. 3) and transferring them to similar holes in immunodiffusion plates (Fig. 4). The concentration peaks of each antibody type were approximated by determining which samples produced reactions of antibody excess when reacted with a standard antigen preparation.

To separate antigens, 3 wells (6 mm in diameter) were cut in a row across the width and at the middle of the agar layer. One well was centered and the others were cut (18 mm center-to-center) on either side of it. A mixture of V and Q was placed in each well, and electrophoresis was continued for 4 hours (4 volts/cm) in a cold room at 10°. The antigens were located by cutting the layer lengthwise into 3 strips so that a well was centered in each (Fig. 5). The strips were transferred to a glass surface and filter papers (140 × 7 × 1 mm) soaked in antiserum were applied to each cut surface. The distance between the paper and well periphery was about 6 mm. Satisfactory precipitation was not obtained by placing filter paper on the surface and near the edge of the agar strip. Strips with fully developed precipitin zones were dried and stained with amido-black or nigrosin (Crowle, 1961).

Density gradient centrifugation. Sucrose columns were prepared with either distilled water or 0.02 *M*, pH 8.0 Tris-HCl buffer. Gradients consisted of 4, 7, 7, and 7 ml of 103.8, 216.2, 338.1, and 470.6 g sucrose per liter solvent, respectively. Gradients were stored overnight at 12° before use. Centrifugation runs with 1 ml of a noninfectious preparation per tube in a Spinco SW 25 rotor were made at approximately 88,000 *g* for 4 or 9½ hours at 6°; rotors were not precooled. Gradients were removed from the tubes in 0.4-ml fractions by gravity flow through a hypodermic needle that punctured the center of the tube bottom. Fractions were numbered consecutively from the bottom and placed in wells of immunodiffusion plates, the wells encircling a central antiserum well. Concentration peaks of each antigen were approximated by determining which fractions gave reactions of antigen excess when reacted with a standard antiserum.

Degradation of V antigen. These tests were similar to those reported by Kelley and Kaesberg (1962) for degradation of alfalfa mosaic virus. From purified preparations of V and of a mixture of V and Q, all of which had been stored at 12° after preparation and contained Tris-HCl buffer and sodium azide, equal portions were taken and mixed with concentrated NaCl solutions to give identical dilutions and NaCl concentrations of 1 *M*, 2 *M*, or 3 *M*. A duplicate of each test portion and a portion of the original preparation were stored at 12° and served as a control. Tubes containing test preparations were placed in a water bath at various temperatures and for varying lengths of time. Relative quantitative assays for V and Q were made by dilution end point titrations.

Particle size determinations. Tremaine and Willison (1961) previously determined the approximate size of the V antigen by a modified double diffusion technique reported by Polson (1958). In the same manner and under similar conditions, tests were

made to determine the approximate size of the Q antigen.

RESULTS

Attempts to Establish V and Q Antigens in Separate Cultures

On the premise that Q might be associated with a virus distinct from V (NRSV), attempts were made to establish V and Q separately in cucumber plants by local lesion transfers and by differential thermal inactivation. *Helianthus annuus* L. plants were inoculated with diluted cucumber sap that contained both antigens. Each transfer to cucumber from the resulting local lesions—and some 100 transfers were made—induced typical necrotic ringspot symptoms and serologically detectable amounts of both V and Q antigens. Neither V or Q was serologically detectable in juice from tissues surrounding the local lesions in sunflower, nor was the juice from these tissues infectious.

In thermal inactivation tests, sap from NRSV-infected cucumber plants was heated to 45°C for 10 minutes. Serological tests with the heated sap indicated that Q had been destroyed but that the concentration of V remained unchanged. Cucumber plants inoculated with the heated sap became infected and contained both the V and Q antigens. The same serological and biological results were obtained with sap heated to 50° or 55°C. When sap was heated above 55°C, however, the V antigen was no longer detectable and the sap lost all infectivity.

Results from the local lesion transfer tests gave further evidence indicating that the two antigens are constantly associated. Results from the thermal inactivation tests indicate that the synthesis of Q is directly dependent upon the multiplication of V. Further, all evidence to date indicates that the V antigen and the infectious particle are the same. However, there is no strong supporting evidence to indicate that the Q antigen is not infectious.

Serological Correspondence between V and Q Antigens

In previous tests (Tremaine and Willison, 1962) V and Q precipitin zones did not contact one another. In most cases, the interaction could not have occurred because adjacent antigen wells contained both antigens. In those few cases, however, where one well contained both antigens and the adjacent well contained only V or where one well contained V and the other Q, the reactant concentrations were such that V and Q zones never extended far enough to meet.

In the present work antiserum was tested against two antigen preparations, one containing V and Q and the other only V. By varying the distance between wells and by adjusting the concentrations of the reactants, the Q zone was extended to the V zone and coalesced with it in a reaction of partial identity (Fig. 1). In no case did a Q zone cross a V zone even though sufficient Q antigen was present to extend the zone at least 3 mm beyond the point of expected intersection with the V zone. Thus, this was the first direct evidence that V and Q antigens were related. Since the Q zone formed nearest to the antiserum well, those antibodies reacting with V presumably could not be Q reactive because the "barrier" effect (Crowle, 1961) of the Q zone would have prevented related antibodies from passing. Therefore, those antibodies re-

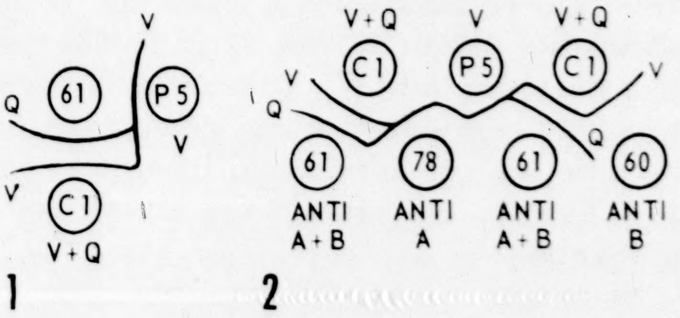

FIG. 1. Drawings of precipitin zone patterns formed by diffusing NRSV antiserum (*61*) against a V antigen preparation from cherry petals (*P5*) and a cucumber preparation (*C1*) containing both V and Q.

FIG. 2. Precipitin zone patterns formed by diffusing two antigen preparations, one from cucumber (*C1*) and one from cherry petals (*P5*), against sera containing A-type (*78*) or B-type (*60*) antibodies or both (*61*). The test shows that A-type serum precipitates both V and Q, whereas the B-type precipitates only V. Note false reaction of identity between V and Q zones in reaction *C1-78-P5*.

sponsible for the coalescence of the V and Q zones were both V and Q reactive.

The existence of two antibody types was confirmed by results from titration tests with sera from weekly bleedings from rabbits injected with a mixture of V and Q. Early bleedings were reactive with both antigens, whereas later bleedings reacted only with V (Fig. 2, serum 60). The latter sera were designated B-type.

To obtain serum containing only the cross-reactive (A-type) antibodies, a rabbit was injected with Q precipitin zones cut from the reaction arena of agar immunodiffusion plates (Allen and Tremaine, 1962). The agar strips were thoroughly washed in distilled water to remove soluble reactants before the strips were used for intramuscular injections. Serum from this rabbit reacted equally well in immunodiffusion tests (Fig. 2, serum *78*) with either V or Q, in contrast to B-type serum that was only V reactive. Zones formed by the precipitation of V and Q from preparation *P5* and *C1*, respectively, with A-type serum (Fig. 2, *C1-78-P5* reaction) coalesced completely, thus falsely indicating a reaction of identity because the complete antibody complement to V was not present to form a spur. Although A-type serum reacts with both V and Q, only a Q zone was formed by the reaction of serum 78 with preparation C1 because A-type antibodies could not pass the zone "barrier" to also precipitate V. This zone, however, probably contained some precipitated V because the particles would have been free to diffuse to the zone in the absence of B antibodies.

Since agar immunodiffusion tests indicated no reactivity between B-type serum and Q antigens, absorption tests were made to check this relationship more thoroughly and to determine whether antibodies specific for Q alone were present in A-type serum. In replicated tests run at 24°C, two increments of B-type serum completely removed V from a mixture of V and Q in which both antigens had the same dilution end points. There was no detectable absorption of Q even after the antiserum concentration was increased to 4 times that required to remove V. The test was repeated at 0° with the final antiserum concentration being 6 times that required to remove V, but again there was no detectable loss of Q.

Absorption tests involving A-type serum and a mixture of V and Q demonstrated the simultaneous precipitation of both antigens. In a similar test where increments of this serum were added to a preparation of V, all of the antigen was eventually removed.

To test for the presence of antibodies specific for Q only, A-type serum was absorbed with increments of a V preparation until V was no longer precipitated as evidenced by its detection in immunodiffusion tests. The absorbed serum was then diffused against a mixture of V and Q. As no precipitin zones appeared, all antibodies capable of reacting with V and Q must have been removed by V. The failure to detect Q-specific antibodies in the A-type absorbed serum may be only apparent, however, since hypoimmune serum was used.

Separation of Antigen and Antibody Types by Electrophoresis and Density Gradient Centrifugation

Since it has been demonstrated that antibodies of differential specificity may have different electrophoretic mobilities in agar (Brown, 1958) serum containing both A and B antibodies was treated in a similar manner. Representative results of these tests (Figs. 3 and 4) show that the two

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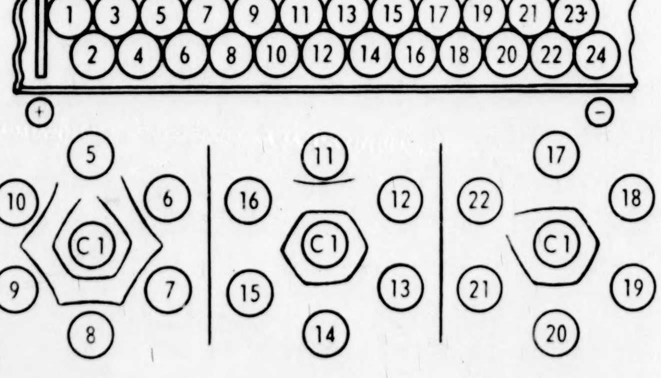

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FIGS. 3 and 4. Agar electrophoresis of antiserum containing A- and B-type antibodies. Figure 3 shows sampling design on electrophorogram, and Fig. 4 shows testing design in immunodiffusion plates. Samples *6–21* contained B-type antibodies, and samples *6–11* contained the A-type.

antibody types have different mobilities. The highest concentration of A antibodies was approximately 24 mm from the origin and that of the B antibodies was approximately 36 mm from the origin. The smaller spread of A antibodies may be explained by their low concentration in the serum used in this experiment.

Under similar conditions, V and Q antigens were separated electrophoretically (Fig. 5). Whereas Q particles did not move from the origin, V particles migrated a mean distance of 24–27 mm, as determined by measurements from the center of the well to the center of the developed precipitin zone.

Replicated density gradient centrifugation runs with a mixture of V and Q demonstrated that the antigens were separable. After 4 hours of centrifugation, Q particles were distributed through samples *38–60* with an approximate concentration peak in samples *54–55*. V particles occurred in samples *37–44* with a concentration peak in samples *41–42*. After 9½ hours of centrifugation, complete separation of V and Q was effected with Q particles occurring in samples *41–56* and having a concentration peak in samples *48–50*. V particles occurred in samples *13–22* with a concentration peak in samples *16–18*. There was no detectable difference either in rate of movement or in distribution of the respective particles in gradients prepared with Tris-HCl buffer as compared with those gradients prepared with distilled water.

Immune Response to V Antigen

Data on titration tests with sera from rabbits immunized with V are presented in Table 1. The results show that V induces both A and B antibodies. In each rabbit, the titer of B antibodies was greater than that of the A-type, and the titration curves of A and B antibodies were similar from rabbit to rabbit. These results correlate positively with those from the absorption tests and indicate that the same determinants, A, are on both V and Q particles.

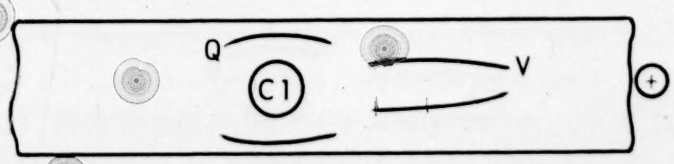


FIG. 5. Drawing of dried agar electrophorogram showing Q precipitin zone at the origin and the V zone near the anode end. Antigens were electrophoresed for 4 hours (4 volts/cm).

TABLE 1

DILUTION END POINTS OF A AND B ANTIBODIES IN SERA FROM THREE RABBITS INJECTED WITH V ANTIGEN[a]

Antigen source	Antibody type	Reciprocal of dilution end point of antibodies in weekly bleedings					
		II	III	IV	V	VI	VII
Petals (P4)	B	128	256	128	128	64	64
	A	2	8	8	8	8	8
Petals (P5)	B	–[b]	128	128	128	64	64
	A	–	8	8	8	8	8
Petals (P6)	B	32	128	128	128	–	64
	A	8	8	8	8	–	4

[a] Purified preparations were made from 140, 74, and 115 g of cherry petals from sources P4, P5, and P6, respectively. The final volume of each was adjusted to give a 20× concentration. Two milliliters of each 20× preparation mixed with adjuvant was used for immunization.

[b] Dash indicates bleedings were not taken.

Degradation of V Antigen under Mild Conditions

Since serological data strongly indicated that Q particles may be an integral part of V at some stage of the disease cycle, attempts were made to form Q by the degradation of V. Initial tests were run at 45° with portions from several preparations containing both V and Q or V alone. Portions were adjusted to either 1 *M*, 2 *M*, or 3 *M* with respect to NaCl. Duplicate controls were held at 12°. During heating, samples for serological testing were taken from each portion after 0, 15, 60, and 120 minutes. The results from the tests on each sample show (Table 2) that although V was heat stable in Tris buffer, these particles degraded when the NaCl concentration was increased. Of more interest was the preservation of Q in 3 *M* NaCl, though the particles were destroyed

TABLE 2

Stability of V and Q Antigens at 45°C as a Function of NaCl Concentration

Preparations[a] of isolate Cl	Treatment	Minutes heated and titer[b] of antigens									
		0		15		60		120		180	
		V	Q	V	Q	V	Q	V	Q	V	Q
1	0.02 *M* Tris	4	4	4	0	4	0	4	0	–[c]	–
	1 *M* NaCl	4	4	3	1	3	0	0	0	–	–
	2 *M* NaCl	4	4	3	3	3	0	0	0	–	–
	3 *M* NaCl	4	4	3	3	3	3	0	3	–	–
2	0.02 *M* Tris	4	0	4	0	4	0	4	0	–	–
	3 *M* NaCl	4	0	4	0	3	0	0	0	–	–
3	0.02 *M* Tris	8	16	–	–	4	0	4	0	2	0
	3 *M* NaCl	8	16	–	–	0	16	0	16	0	16

[a] Prepared from cucumber by clarification and differential centrifugation on three different occasions.
[b] Titer expressed as reciprocal of dilution end point.
[c] Dash indicates assays were not made.

in 0.02 *M* Tris even after 15 minutes of heating. There was no evidence that Q was formed as V was degraded at this temperature.

When the refrigerated control portions were tested 2 weeks after storage at 12°, trace amounts of Q were detected in those portions originally containing only V and 3 *M* NaCl. This was the first direct evidence, therefore, that Q was a structural part of V. The results further indicated that the successful degradation of V required milder conditions than had been thought initially.

Daily assays of a V preparation containing 3 *M* NaCl and held at room temperature showed that over a 28-day period the titer of V dropped from 1:32 to 1:8 and the titer of Q increased to 1:4. Degradation of V into Q was accelerated by incubation at 30° or 35°. For example, samples taken from the above preparation after 20 days had V and Q titers of 1:16 and 1:2, respectively. When the samples were heated for 90 minutes at 35°, the titers of V and Q changed to 1:8 and 1:4, respectively. Additional tests showed that the degradation of V into Q at room temperature was faster in 4 *M* than in 3 *M* NaCl.

Microbial action is not believed to be responsible for the degradation of V into Q since all test and control preparations contained 0.015 *M* sodium azide. Further, none of the preparations became cloudy or developed odors common to contaminated preparations. Careful observation of the titration tests in immunodiffusion plates also indicated no contamination.

Apparent Heterogeneity among Q Antigens

A survey of immunodiffusion tests involving comparisons among antigen preparations of some 20 or more different NRSV isolates and their respective antisera has revealed only reactions of identity between V antigens, i.e., no spurs were formed after the V zones of different virus isolates had coalesced. Hence, B determinants on all virus isolates appeared to be homogeneous. In several cases, however, reactions of partial identity occurred between Q zones where several different virus isolates were tested against 1 antiserum (Fig. 6). Serum 25, therefore, apparently contained at least two types of A antibodies, and the determinant for one of these types was absent on Q particles in isolate C2 and C3. Thus, the differentiating A antibodies, i.e., those that apparently reacted only with Q particles in preparation C1, were free to pass through the developing Q zones of isolates C2 and C3 to form spurs on the Q zone of C1.

In another case, in which several heterologous antisera were tested against a virus

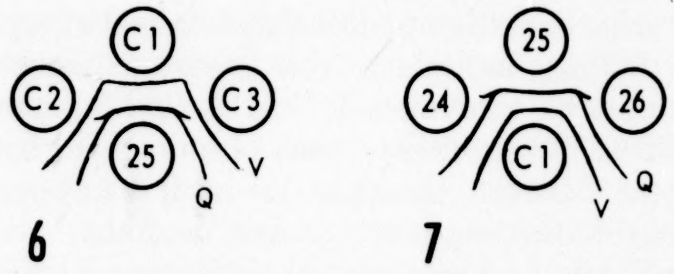
isolate, a reaction of partial identity was observed between Q particles in the same isolate (Fig. 7). Those Q particles that apparently were unreactive with sera 24 and 26 were free to pass through the Q zones formed by these sera to react with that fraction of A antibodies apparently peculiar to serum 25.

These results were from routine tests in which the effect of reactant concentrations was not studied. Other possible interpretations of these results will be presented in the discussion.

Particle Size Determination of Q Antigen

In previous work Tremaine and Willison (1961) calculated the approximate particle size of V (30 ± 10 mμ) by a modified immunodiffusion technique reported by Polson (1958). Size determinations for Q were desired in the present study since the information would indicate whether Q approached the size of a virus subunit or capsomere.

The results of the diffusion tests with Q are presented in Table 3 with those published for V. The sharpest Q precipitin zones were formed at antigen dilutions of 1:4 and 1:8. With a precision of measurement of 0.5 mm, the X_g value (the distance to the sharpest zone from the antigen meniscus) for Q was approximated to be 9.5 ± 0.5 mm. Using this value in Polson's equation, the diffusion coefficient of Q was calculated to be 1.0 $(\pm .3) \times 10^{-7}$ cm^2 sec^{-1}. Assuming the particles to be spherical, the diameter was calculated to be 4(±1) mμ by substituting the diffusion coefficient of Q in the Stokes-Einstein equation.



FIGS. 6 and 7. Drawing of precipitin zone patterns showing heterogeneity among Q antigens. Figure 6 shows reaction of partial identity among Q antigens from three different isolates (*C2*, *C1*, and *C3*). Figure 7 shows reaction of partial identity among Q antigens in the same isolate (*C1*).

DISCUSSION

The results of these studies indicate that Q particles are at some stage of the disease cycle an integral part of V (NRSV) and are apparently morphological subunits or capsomeres (Caspar *et al.*, 1962). Similar virus-related particles also considered to be naturally occurring, noninfectious, and considerably smaller than the virus have been reported for other viruses, e.g., foot-and-mouth disease (Cartwright, 1962), tobacco mosaic (Takahashi and Gold, 1960), and brome mosaic (Hamilton, 1961) viruses. Takahashi and Gold (1960) working with TMV and X protein and Cartwright (1962)

TABLE 3

POSITION AND WIDTH OF V AND Q PRECIPITIN ZONES AS A FUNCTION OF ANTIGEN DILUTION

Antigen dilution	1:1	1:2	1:4	1:8	1:16	1:32	1:64
V							
Distance from antigen meniscus (mm)	ND[b]	ND	7	6	5	4	ND
Width[a] of zone	+++	+++	++	+	+	++	+++
Q							
Distance from antigen meniscus (mm)	ND	11	10	9	8	ND	ND
Width of zone	+++	++	+	+	++	+++	+++

[a] The width of precipitin zones was rated visually into three categories: +++, broad; ++, medium; +, sharp.

[b] ND indicates measurement not made.

working with FMDV and its accompanying 7-mμ particle demonstrated the same serological relationships between the respective particles that we have shown between V and Q, viz., that the virus has at least two different types of determinants, one which is peculiar to the virus and one which is common to both virus and subunits. Takahashi and Gold (1960) and Aach (1959) were able to present good evidence that the determinant peculiar to the intact virus is formed by contributions from adjacent subunits since on polymerization of subunits the determinant was again detectable. Whether this determinant on TMV, FMDV, and NRSV is analogous to an intersubunit bond is not known, but it would seem that the determinants represent at least part of the bonding system if, as with TMV, they can be disrupted and then reformed by subunit polymerization.

The reactions of partial identity between Q particles of different isolates (Fig. 6) has been explained by assuming that serum 25 contained two types of A antibodies and that the determinant for one of these types was absent on Q particles in preparations of isolates C2 and C3. An alternative explanation might be that the spurs do not represent strain differences but only concentration differences between two Q species that are present in each isolate, i.e., the Q species involved in the spur formation is also present in C2 and C3 but below the level of detection. The results might also be explained by assuming that both the A antibody and the Q antigen populations, respectively, are homogeneous rather than heterogeneous and that the concentration of Q particles in C2 and C3 is much lower than in C1. Excess antibodies, therefore, would diffuse through the areas of lowest antigen concentration, viz., in the areas of zone coalescence, and precipitate with antigen from C1 thus forming spurs that falsely indicate partial identity (Crowle, 1961).

The interpretation of the reactions of partial identity in Fig. 7 was that the preparation of isolate C1 contained a heterogeneous but partially related Q population and that those Q particles unreactive with sera 24 and 26 passed the Q zones formed by these sera and reacted with a fraction of the A antibodies peculiar only to serum 25. Alternative explanations which take into account concentration differences must, however, be considered. For example, all sera may have the same types of A antibodies but one type is below the level of detection in sera 24 and 26, therefore allowing part of one Q type to diffuse until precipitated by antibodies from serum 25, thus forming spurs. Or, if the Q antigen and the A antibody populations, respectively, are instead homogeneous, then spur formation might be caused by low antibody titers in sera 24 and 26 as compared with 25, thus allowing excess Q particles to diffuse until precipitated by A antibodies from serum 25 (Crowle, 1961).

The only evidence to indicate that the spur formation in Figs. 6 and 7 was not due to concentration differences was that the Q precipitin zones apparently remained sharp, a finding indicative that reactant ratios were near equivalence. Further, no faint or diffuse precipitation was observed in the areas between the Q zones and the spurs, thus indicating that excess reactant, i.e., antibody in Fig. 6 and Q antigen in Fig. 7, had not passed through the zones to be precipitated as spurs and to give a false reaction of partial identity.

The results that indicate that the Q population in isolate C1 (Fig. 7) is serologically heterogeneous though related do not agree with expectations if the present views on virus structure are considered, viz., that subunits of a given virus are structurally and chemically identical (Caspar and Klug, 1962). The same results, moreover, were reported by Tremaine and Willison (1962), who used different NRSV isolates and antisera. Similar and more extensive serological evidence indicating that virus subunits may not be antigenically identical was reported by Kleczkowski for TMV (1957, 1961). He apparently demonstrated the presence of at least six types of antigenic determinants, each occurring on a different protein subunit or polymer. By amino acid analysis of TMV protein, Aach (1960) was also able to present evidence indicating

that subunits of a virus particle are different.

An alternative explanation of these results is that the plants from which these NRSV and TMV isolates were extracted were infected with a mixture of mutants or strains each contributing distinct but serologically related subunits. Aach, however, was unable to find evidence that his isolates contained mixtures of strains. It may be wise to keep open the question concerning homogeneity of virus subunits since information on this point is pertinent to a better understanding of virus synthesis.

The results from density gradient centrifugation studies with mixtures of V and Q, and those from tests involving the injection of rabbits with preparations of pure V, indicate why the Q immune system, i.e., Q particles and A antibodies, was not detected in previous work by the senior author (Allen, 1964). In that work, antigen preparations were made from small quantities of cucumber tissue, thus the initial concentrations of both V and Q were low. Additional reduction in the concentration of Q to the marginal level of detection would have resulted from density gradient centrifugation since only the infectious zone was selected for concentration and injection into rabbits. Concentration was accomplished by centrifugation for 1½ hours at about 145,000 *g*, which would pellet only a small part of the Q that survived the density gradient separation. Therefore, it is apparent that the rabbits received preparations of almost pure V. Since the titer of B antibodies that was induced was no greater than 1:4, extrapolation from our present results (Table 1) indicates that A antibodies, if present, would have been below the level of detection and explains the failure of Allen's sera to precipitate Q particles in immunodiffusion tests.

It is commonly stated that when hyperimmune sera, i.e., sera with high collective antibody titer, is induced, the "specificity" is reduced as the titer increases (Boyd, 1956; Crowle, 1961; Matthews, 1957). This experience with hyperimmune sera may be explained by assuming that as the titer increases nondifferentiating antibodies become more prevalent and increase disproportionately to differentiating antibodies so that the participation of the latter type in immune reactions is less obvious. This being the case, the term "specific" implies a quantitative change in antibody types during the response phase and not a qualitative change in those antibodies already formed or the formation of new antibody types. It is probable, however, that a qualitative change in antibody types also may occur as immunization progresses, the antibodies produced later being either differentiating or nondifferentiating types.

The correlation of "specificity" with hypo- or hyperimmune NRSV antisera may not be constant because several antibody types are induced and the titer of each is dependent on the antigen, V or Q, used for injection. To our knowledge, the B antibody group is comprised of no differentiating types; whereas, the A group may contain them in addition to nondifferentiating types. If rabbits are injected with V alone, a low dose will induce only B antibodies and, therefore, nondifferentiating serum. If rabbits are given a high dose of V, there may be sufficient A antibodies induced, in addition to the B type, to produce differentiating serum. If the rabbit receives both V and Q or Q alone, the total antigen dose required to induce A antibodies will be lower than with V alone since A determinants may equal B determinants or be the only ones introduced.

The particle size determined for Q indicates that these particles are small enough to be considered as capsomeres (Bils and Hall, 1962; Cartwright, 1962; Horne and Nagington, 1959; Pereira *et al.*, 1963). Since Q particles were assumed to be spherical in these calculations and since the present information (Lafferty and Oertelis, 1963; Noyes, 1962) suggests that capsomeres may be slightly ellipsoidal, it is possible that Q particles are slightly smaller than 4(±) mμ.

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...Synthesis and Distribution of Tobacco ...Antigen in Tobacco[1]

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...September 4, 1964

...ining fluorescein-conjugated antibodies that ...us (TMV) antigen in plant tissues. Mesophyll ...and paraffin sections of leaves were stained ...ly the synthesis, distribution, and movement ...er infection either with TMV or with its ribo- ...or by infectivity assays on *Nicotiana tabacum* ...ours) when samples were obtained at 6-hour intervals. TMV antigen was first noticed in infected mesophyll cells in the form of tiny specks of stained material which later formed a ring around the chloroplasts. No antigen was noticed within chloroplasts or nuclei of infected cells. All cell types except the primary xylem elements were susceptible to TMV infection; however, in systemically infected leaves, some mesophyll cells in the dark green areas appeared to be resistant to virus invasion. In stems adjacent to inoculated leaves, TMV antigen was first detected within the phloem and secondary xylem. In stem tips of infected plants, cells containing virus antigen were unevenly distributed. It was demonstrated that a strain of TMV hitherto believed to be a "proteinless" mutant produced TMV protein in infected cells.

INTRODUCTION

The biological behavior of plant viruses within infected cells and tissues has been studied by the use of infectivity assays (Bennett, 1956), staining methods (Bald, 1949a, b; Hirai and Wildman, 1963) ultraviolet and phase contrast microscopy (Zech, 1954; Bald, 1964a,b), and electron microscopy (Shalla, 1959). While the above methods have given very useful information, not all the methods are specific to viral protein or viral nucleic acid. The fluorescent antibody method of Coons *et al.* (1942), which utilizes fluorescein-conjugated antibodies as specific stains for viral protein, is potentially a very useful tool for obtaining detailed and more direct information on tissue and intracellular localization of plant viruses, but so far it has found only limited application in the study of virus infections in plants (Schramm and Röttger, 1959; Day and Venables, 1961; Nagaraj and Black, 1961; Nagaraj, 1962; Worley and Schneider, 1963). This paper presents the results of immunofluorescence studies on the synthesis, movement, and distribution of tobacco mosaic virus (TMV) in *Nicotiana tabacum* L. var. Turkish.

[1] This investigation was supported in part by Public Health Service research grant CA 02245 from the National Cancer Institute, Public Health Service, and United States Public Health Service training grant 5 T4 CA 5028.

[2] Present address: F.A.O. Plant Virologist, c/o UNTAB, P.O. Box 1864, Manila, Philippines.

MATERIALS AND METHODS

Virus. The common strain of TMV was used throughout the work unless mentioned otherwise; purified preparations of TMV were kindly supplied by Dr. C. A. Knight and were stored at 4°C as concentrated solutions in water (about 30 mg/ml) with a few drops of chloroform.

Fulton for supplying a NRSV isolate (isolate G) and homologous antiserum.

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Immunofluorescence Studies on Synthesis and Distribution of Tobacco Mosaic Virus Antigen in Tobacco[1]

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Techniques were improved for obtaining fluorescein-conjugated antibodies that specifically stained tobacco mossaic virus (TMV) antigen in plant tissues. Mesophyll cells separated by pectinase digestion and paraffin sections of leaves were stained with such fluorescent antibodies to study the synthesis, distribution, and movement of TMV antigen. The latent periods after infection either with TMV or with its ribonucleic acid, as studied antigenically, or by infectivity assays on *Nicotiana tabacum* var. Xanthi-nc, were similar (18–24 hours) when samples were obtained at 6-hour intervals. TMV antigen was first noticed in infected mesophyll cells in the form of tiny specks of stained material which later formed a ring around the chloroplasts. No antigen was noticed within chloroplasts or nuclei of infected cells. All cell types except the primary xylem elements were susceptible to TMV infection; however, in systemically infected leaves, some mesophyll cells in the dark green areas appeared to be resistant to virus invasion. In stems adjacent to inoculated leaves, TMV antigen was first detected within the phloem and secondary xylem. In stem tips of infected plants, cells containing virus antigen were unevenly distributed. It was demonstrated that a strain of TMV hitherto believed to be a "proteinless" mutant produced TMV protein in infected cells.

INTRODUCTION

The biological behavior of plant viruses within infected cells and tissues has been studied by the use of infectivity assays (Bennett, 1956), staining methods (Bald, 1949a, b; Hirai and Wildman, 1963) ultraviolet and phase contrast microscopy (Zech, 1954; Bald, 1964a,b), and electron microscopy (Shalla, 1959). While the above methods have given very useful information, not all the methods are specific to viral protein or viral nucleic acid. The fluorescent antibody method of Coons *et al.* (1942), which utilizes fluorescein-conjugated antibodies as specific stains for viral protein, is potentially a very useful tool for obtaining detailed and more direct information on tissue and intracellular localization of plant viruses, but so far it has found only limited application in the study of virus infections in plants (Schramm and Röttger, 1959; Day and Venables, 1961; Nagaraj and Black, 1961; Nagaraj, 1962; Worley and Schneider, 1963). This paper presents the results of immunofluorescence studies on the synthesis, movement, and distribution of tobacco mosaic virus (TMV) in *Nicotiana tabacum* L. var. Turkish.

MATERIALS AND METHODS

Virus. The common strain of TMV was used throughout the work unless mentioned otherwise; purified preparations of TMV were kindly supplied by Dr. C. A. Knight and were stored at 4°C as concentrated solutions in water (about 30 mg/ml) with a few drops of chloroform.

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Plants. Turkish tobacco plants were grown in an insect-proof greenhouse in which, during the period of this work, the temperature ranged approximately from 22° to 30°C. Plants having 4 or 5 expanded leaves (approximately 7 × 5 cm) were used for inoculating TMV unless otherwise specified. After inoculation the test plants and controls were placed in a temperature controlled chamber (21°–23°) illuminated with fluorescent light day and night. *Nicotiana tabacum* var. xanthi-nc was used for the infectivity assays by local lesion production.

Antiserum. The methods for producing TMV antisera and measuring their titers were the same as described earlier (Nagaraj, 1962). Antisera from 7 rabbits, having titers between 1/1024 and 1/2048, were used for preparing fluorescent antibodies and they gave similar results.

Preparation and purification of conjugates. The methods for preparing and purifying the conjugates were essentially the same as described earlier (Nagaraj, 1962) except for the following modifications: (1) Saturated $(NH_4)_2SO_4$ was brought to pH 7.0 with 1 *M* NaOH and then used for precipitating globulins. This treatment caused less denaturation of proteins than when the pH was unadjusted. (2) Between the two concentrations of fluorescein isothiocyanate[3] studied, namely 20 and 40 mg per gram of globulin, the former was better in that the conjugate could be stored longer with less precipitation on thawing and hence was used throughout this work.

Nonspecific staining of the plant tissues by conjugates was satisfactorily removed by two 30-minute absorptions with 50 mg of mouse liver powder per milliliter, which was suspended in PBS (0.01 *M* potassium phosphate buffer containing 0.15 *M* saline) and centrifuged before absorption, and one 30-minute absorption with HeLa cells at 5×10^6 cells per milliliter of conjugate.

Storage of conjugates. The traditional method of storing conjugates at 4° after addition of 1:10,000 merthiolate (Coons, 1958) was found to be unsatisfactory. Absorbed conjugates stored even for 18 hours at 4° (with or without merthiolate) caused nonspecific staining. Storing such conjugates at −40° without merthiolate was found to be a satisfactory method which preserved their specificity for a period of 1–1½ months. After this period the conjugates became nonspecific, but the specificity could be restored by passing them through Sephadex (Nagaraj, 1962) to remove free fluorescein. It was desirable not to refreeze the conjugates once thawed, as repeated freezing and thawing increased nonspecific staining. Conjugates were, therefore, stored in quantities of 0.5–1 ml.

Numbering of leaves. Unless mentioned otherwise, leaves of experimental plants were numbered consecutively from the tip downward, leaf number 1 being the youngest discernible leaf.

Separation of mesophyll cells. For separating mesophyll cells, leaves (excluding midribs) were cut into 1 × 2-mm pieces and digested by shaking them for 3¾ hours at 37° with 0.2% pectinase (Zaitlin, 1959) dissolved in most experiments in 0.1 *M* Sorensen's phosphate buffer at pH 5.9 containing 0.35 M sucrose. The cells were centrifuged in conical centrifuge tubes at 1000 *g* for 5 minutes and washed twice with buffer *minus* pectinase.

Under the above conditions of digestion a large number of cells could be separated. However, in many experiments the cells were plasmolyzed and the degree of plasmolysis was found to vary with age of leaves. Usually the cells of the second expanded leaf from the top of a 5-leaf plant were least plasmolyzed. Therefore, in studies on the time sequence of appearance of TMV antigen this leaf was inoculated and digested at various periods after inoculation.

In some experiments water was used as a solvent for pectinase, and tissues were digested at 24°. Under these conditions plasmolysis was negligible and nuclei were seen more often than in cells separated by the usual method, but far fewer cells were obtained from the same amount of leaf tissue.

Frozen sections. Frozen sections of tobacco stems were cut in a cryostat using a glass

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guide, thawed, and dried as described by Coons *et al.* (1951). The temperature of the cryostat was a critical factor in obtaining good sections. Sections 20–25 μ in thickness could be easily cut at −12 to −15°. The sections were mounted on slides smeared with a modification of Haupt's adhesive (Schramm and Röttger, 1959) and stored at 4° until ready for staining. The adherence of the sections to the slides was good even if they were stored for a week before staining.

Satisfactory thin frozen sections (10–15 μ) of leaves could not be cut even when leaf material was embedded in gelatin as suggested by Schramm and Röttger (1959).

Paraffin sections. For obtaining leaf sections, without loss of antigenicity, a modification of the paraffin embedding procedure of Saintemarie (1962) was used. The following is a brief description of the embedding procedure: The leaf material was cut into pieces 3 × 10 mm, dehydrated in three hourly changes of ice-cold absolute alcohol with overnight storage in the last change. It was then passed through three changes of ice-cold xylol, and was infiltrated with three changes of Tissuemat for 9–10 hours at 65°, blocked, and stored at 4°. The sections were cut at 5 μ, mounted on slides smeared with the modified Haupt's adhesive, stretched in water at about 40°, dried for 30 minutes at 37°, and stored at 4°. Ribbons prepared by this procedure could be stored for over 70 days without adversely affecting specific fluorescent antibody staining.

Fixation and staining. (a) *Separated cells:* Cells were fixed for 5 minutes in absolute alcohol in a conical centrifuge tube, washed in PBS by centrifugation at 1000 *g* for 5 minutes, and kept suspended in a few drops of absorbed conjugate for 30 minutes. The tubes were then filled with PBS and centrifuged. The washing in PBS was repeated and the cells were suspended in a solution of 7 parts by volume of glycerin buffered with 3 parts of PBS (buffered glycerin). (b) *Frozen sections:* Sections were fixed in absolute alcohol for 15 minutes and dried at 37° for 30 minutes. Three drops of absorbed conjugate were layered on each section for 30 minutes in a moist chamber; the section was washed with PBS for 15 minutes, and mounted in buffered glycerin. (c) *Paraffin sections:* After deparaffinizing for 30 minutes in 2 changes of xylol, the sections were passed through two changes of absolute alcohol for a total period of 15 minutes, dried for 30 minutes at 37°, stained, and mounted as mentioned under frozen sections.

Controls of specificity. To make sure that staining by TMV antiserum conjugate was specific to TMV antigen, the following controls were frequently included: (1) TMV-infected sections or cells were treated with fluorescein-conjugated poliovirus antiserum (kindly supplied by Dr. T. H. Dunnebacke) or conjugated turnip yellow mosaic virus antiserum. (2) TMV-infected cells and sections were first treated with unconjugated TMV antiserum for 30 minutes (three changes), and then with TMV antiserum conjugate. (3) Healthy tobacco cells and sections comparable to the infected ones were also treated with TMV antiserum conjugate, and other conjugates as in (1).

Complete specific inhibition with TMV antiserum was obtained with 5-μ paraffin sections, but with cells and 25-μ frozen sections the inhibition was sometimes not complete. In such cases if controls (1) and (3) were negative it was concluded that staining was specific to TMV antigen.

Definitions. Cells and tissues which showed staining specific to TMV antigen, will be referred to as infected even though it is possible that some of these cells or tissues (enucleate sieve tubes for example) may not have supported virus multiplication and may have contained antigen transported from adjacent infected cells.

The term "latent period" will be used as defined by Yarwood (1952).

RESULTS

Antigen in Leaves

Inoculated leaves: In three preliminary experiments the second expanded leaf from the top of a 5-leaf plant was inoculated. The first sampling was done immediately after inoculation (0 hour). Inoculated plants were then kept under 16 hours light and 8

hours darkness. Inoculated leaves from comparable plants were cut at 20, 42, and 66 hours after inoculation and the cells were separated by pectinase digestion, stained, and examined. TMV antigen was first noticed only at 42 and 66 hours after inoculation, but not at 0 and 20 hours.

In the next three experiments the inoculated plants were kept under constant illumination at 22 ± 1°. The inoculation times were so arranged as to give 0, 6, 12, 18, 24, 30, 36, 42, and 48-hour samples on the same day. One series of plants was inoculated with TMV, and another with TMV ribonucleic acid (RNA) prepared by phenol extraction (Gierer and Schramm, 1956). All these samples and the controls were digested with pectinase, and the separated cells were stained and examined. In each experiment TMV antigen was first detected in TMV- and RNA-inoculated leaves at the same time after inoculation, namely, 18 or 24 hours. In two experiments TMV antigen was first detected only in samples collected at 24 hours and later. In one experiment antigen was detected in samples collected at 18 hours and later. Although quantitative measurements were not made, general observations indicated that there was a gradual increase in the number of infected cells with time.

In separated cells the intracellular localization of antigen was not very clear. When chloroplasts could be clearly seen, no antigen was observed within them.

In 5 experiments inoculated leaves were cut and embedded in paraffin at 0, 6, 12 18, 24, 30, 36, 42, and 48 hours after inoculation. At 0, 6, and 12 hours only the inoculated virus could be detected on the epidermal cells. The earliest period at which antigen could be detected in paraffin sections varied between 18 and 36 hours from experiment to experiment. Also, when antigen was detected in some samples at 24 hours, it was not always detected in others in the same experiment at 30 hours, 36 hours, and so on, clearly indicating an inadequate sampling.

In paraffin sections intracellular localization was very clear. In some cells in early stages of infection (18–24 hours after inoculation) antigen was found in the form of tiny specks apparently on the outside of the chloroplasts in mesophyll cells, or within the cytoplasm of epidermal cells. At this early time it was not possible to photograph these specks in color because their fluorescence rapidly faded under ultraviolet light. A few hours later antigen was detected in some cells in larger masses which were much smaller in size than the chloroplasts (Fig. 1). In other cells, antigen was found in the form of partial or complete rings around the chloroplasts (Fig. 2). In later stages of infection, particularly in systemically infected leaves, antigen was seen in the form of large distinctive masses protruding into, but infrequently filling, the vacuole of the cell; the vacuoles of the cells were mostly unstained, or faintly stained (Figs. 3 and 4). In most cases chloroplasts did not appear to contain any antigen within them. However, in some cases large masses of antigen appeared to lie over chloroplasts, and in very rare cases sections of one or two chloroplasts appeared to be full of antigen. The nucleus, whenever recognizable, did not have antigen either within or around it.

All cell types in the leaf appeared to be

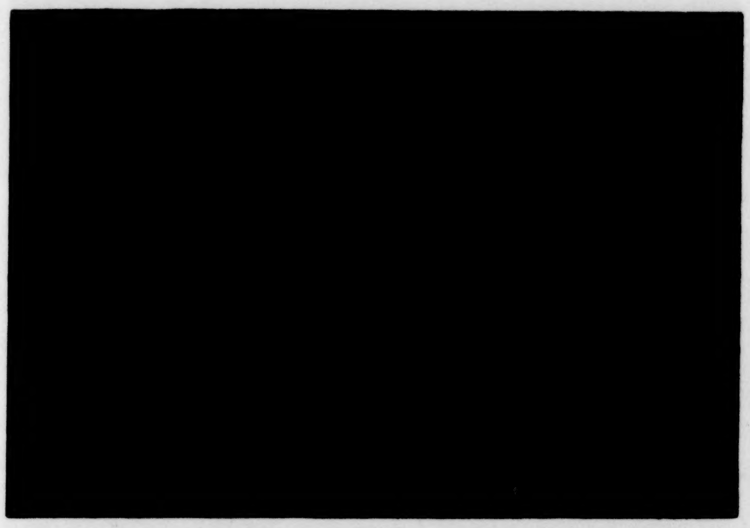
FIGS. 1–4. Photographs of cross sections of tobacco leaves. The blue fluorescence seen in all sections is the autofluorescence of tobacco tissues. TMV antigen-containing areas show yellow-green fluorescence. Magnification: ×160.

FIG. 1. Antigen can be seen as tiny specks outside of chloroplasts; 24 hours after TMV inoculation.

FIG. 2. Antigen is seen around chloroplasts 30 hours after inoculation with TMV.

FIG. 3. Systemically infected leaf (one month after appearance of symptoms) showing viral antigen in large masses and crystals.

FIG. 4. Systemically infected leaf showing antigen in large masses. In the vacuoles, antigen either is absent or is present in small quantities when it is apparently released by the rupture of large masses of antigen.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Внесение удобрений под яровую пшеницу при нормальном водоснабжении увеличивает урожай на 240%, а при избыточном увлажнении — только на 134%. В условиях переувлажнения эффект минеральных удобрений был почти в два раза ниже.

Очень важно установить, как влияют удобрения на устойчивость растений к избыточному увлажнению. Трехлетние опыты (табл. 6) показали, что яровая пшеница на фоне полного минерального удобрения развивается значительно лучше и дает урожай выше, чем на неудобренном фоне даже при переувлажнении. Однако степень снижения урожая зерна яровой пшеницы на удобренном фоне во все фазы развития растений больше, чем на неудобренном.

Объясняется это тем, что у растений на фоне с удобрениями лучше развиты листовая поверхность, кустистость и другие ценные хозяйственные признаки. Такие хорошо развитые растения, попав в неблагоприятные условия переувлажнения, страдают значительно сильнее, чем слаборазвитые.

SUMMARY

The surplus soil moisture is not desirable at all stages of the development of crops; it suppresses the growth of vegetative and reproductive organs and decreases yields.

Cereals (wheat, barley, oats) are less resistant to surplus soil moisture at the shooting stage, the decrease of the yield amounting to 71 per cent. Spring wheat suffers from overmoisted soils more than other crops.

ZUSAMMENFASSUNG

Übermäβige Bodenfeuchtigkeit ist in allen Phasen der pflanzlichen Entwicklung schädlich; sie unterdrückt das Wachstum der Vegetativ-und Reproduktionsorgane und senkt die Erträge.

Die Getreidekulturen (Weizen, Gerste, Hafer) sind am wenigsten resistenzfähig gegenüber der übermäβigen Bodenfeuchtigkeit in der Phase der Halmbildung. Die Körnererträge sinken auf 71% herab. Am stärksten leidet unter übermäβiger Bodenfeuchtigkeit der Sommerweizen.

RESUME

L'humidité abondante du sol est nuisible dans toutes les phases de développement des plantes. Elle accable la croissance des organs végétatifs et reproductifs et réduit le rendement.

Les céréales (blé, orge, avoine) sont moins résistantes à l'humidification abondande dans la phase de montaison. La récolte se réduit jusqu' à 71%. L'humidification abondante exerce la plus mauvaise action sur le blé de printemps.

Новые книги

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ВЛИЯНИЕ БУРОЙ ЛИСТОВОЙ РЖАВЧИНЫ НА ДЫХАНИЕ ПШЕНИЦЫ

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В НАСТОЯЩЕЕ время доказана роль окислительных процессов при поражении растений микроорганизмами (2—6), что ярко выражается в усилении интенсивности дыхания (1, 12, 14, 18).

Исследования Шоу и Самборского (22), проведенные с пшеницей, зараженной стеблевой ржавчиной (Puccinia graminis), показали, что повышение уровня дыхания наблюдалось у растений восприимчивых и устойчивых сортов, причем у первых на 50—60% выше, что особенно ясно проявляется в период наиболее быстрого роста гриба. Аллен (7) пришел к выводу, что эти изменения при заражении обусловлены деятельностью клеток растения-хозяина, которые активно окисляют углеводы в период вегетативного роста паразита, так как дыхательный коэффициент близок к 1,0; после этого интенсивность дыхания и рост паразита начинают уменьшаться.

Известно, что уровень дыхания пораженных стеблевой ржавчиной растений пшеницы может быть в 2—3 раза выше, чем у здоровых растений. Степень возрастания зависит от целого ряда причин, в том числе от условий выращивания растений и от количества вносимого заразного начала.

Остается до сих пор недостаточно изученным характер специфического воздействия, оказываемого ржавчинными грибами на окислительную систему растения-хозяина. Имеющиеся по этому вопросу в литературе данные указывают, что различия между дыханием больной и здоровой ткани касаются не только интенсивности процесса, но и участвующих в нем ферментных систем. Отсюда становится очевидной важность выяснения каталитических механизмов, регулирующих дыхание тканей пораженного растения. Изучение характера качественных сдвигов в дыхании и изменения доли участия отдельных ферментных систем могло бы приблизить нас к пониманию самых интимных сторон взаимодействия гриба и растения-хозяина.

Представляют особый интерес работы с использованием меченых радиоактивных сахаров (8, 9, 23, 24). Сравнение скорости выделения радиоактивной CO_2 из листьев, получавших глюкозу, меченную по углероду в положении C_1 и C_6, дало возможность установить, что, хотя на стадии хлорозных пятен интенсивность дыхания возрастает, но без сколько-нибудь значительных качественных изменений в ферментативных системах (8). Дальнейшее увеличение уровня дыхания, совпадающее с процессом споруляции, характеризуется падением величины отношения C_6/C_1, что, возможно, связано с возрастанием апотомического шунта дыхания при заражении растений облигатными паразитами. В дальнейших работах подтвердилось, что в пораженной ржавчиной пшенице возрастает активность двух дегидрогеназ пентозофосфатного цикла: дегидрогеназы глюкозо-6-фосфата и 6-фосфо-глюконовой кислоты (13).

Для изучения путей дыхания применяются также дыхательные яды избирательного действия. Несмотря на относительность данного метода, поскольку применяемые ингибиторы малоспецифичны, он до сих пор остается важным вспомогательным средством исследования отдельных звеньев метаболизма.

Мы использовали фторид натрия, азид калия, монойодуксусную и малоновую кислоты. Фторид ингибирует активность одного из ферментов гликолиза — энолазы, образуя неактивный комплекс энзиммагний — фторфосфат; монойодуксусная кислота — один из основных тиоловых реагентов — блокирует дегидрогеназу фосфоглицеринальдегида, катализирующей одну из узловых окислительных реакций данного пути; азид калия дает прочные комплексы с некоторыми металлами простетической группы ряда оксидаз — цитохромоксидазы, пероксидазы и других.

В качестве ингибитора цикла трикарбоновых кислот была использована малоновая кислота, которая действует на сукцинатдегидрогеназу (21). Она содержит две карбоксильные группы и имеет сходную с сукцинатом структуру, и ее влияние в основном определяется соотношением сукцинат: малонат (20).

В опытах использовали два вида пшеницы, отличающихся по реакции на поражение бурой ржавчиной (Puccinia triticina). Поражаемость определяли по шкале Джексона и Мэйнса (19): для Triticum timopheevi Zhuk оценка была ноль, для Triticum persicum — четыре. Семена этих видов пшеницы получали из коллекции ВИРа.

Растения выращивали в почвенной культуре в теплице при контролируемых условиях освещенности, температуры и влажности. Первые листья шестидневных проростков инокулировали смесью уредоспор бурой ржавчины (раса 77) и талька, помещали на сутки во влажную камеру. Контрольные растения того же возраста опыливали только тальком и ставили в идентичные условия.

Мы изучали характер изменения путей дыхания, вызванного инфекцией, на различных стадиях развития гриба. Поскольку в ранее опубликованных биохимических исследованиях пораженных ржавчиной растений пшеницы об отдельных этапах развития гриба не упоминалось, считаем необходимым кратко описать их.

Первые симптомы поражения ржавчиной в наших условиях становятся заметными на четвертый день после инокуляции — это стадия первых хлорозных пятен. На седьмой день на листьях восприимчивого вида (Tr. persicum) начинают образовываться уредопустулы. Растения устойчивого вида (Tr. timopheevi) обнаружили характерную реакцию «сверхчувствительности» на внедрение паразита. В местах прорастания уредоспор образуются некрозы, хорошо видимые на седьмой день. На одиннадцатый день происходит активная споруляция гриба на листьях растений восприимчивого вида.

Таблица 1

Характеристика дыхания первых листьев здоровых десятидневных растений, устойчивых и восприимчивых к ржавчине видов пшеницы (Q_{O_2} на 10 мг сухого веса в час)

Вид пшеницы / Вводимое соединение, концентрация его	Tr. persicum		Tr. timopheevi	
	интенсивность дыхания	процент ингибирования дыхания	интенсивность дыхания	процент ингибирования дыхания
Фосфатный буфер (pH — 7,0) — контроль	109,5	—	85,6	—
Фторид натрия, $6\cdot10^{-2}$ М	33,6	69	20,5	76
Монойодацетат, $1\cdot10^{-2}$ М	42,7	61	38,8	55
Азид калия, $5\cdot10^{-3}$ М	111,3	+2*	76,9	10
Малонат, $1\cdot10^{-2}$ М	37,5	66	43,2	49

* Наблюдается стимулирование поглощения кислорода.

Пробы брали с первых листьев утром на 4, 7 и 11 день после нанесения инфекции. Работу проводили на промытых водой и подсушенных срезах листьев толщиной в 1,0—1,5 мм. Интенсивность дыхания определяли по поглощению кислорода манометрическим методом на аппарате Варбурга и выражали в микролитрах кислорода на 10 мг сухого веса листьев в час. Растворы ингибиторов готовили на фосфатном буфере с pH, оптимальным для действия каждого из применяемых ядов.

Мы считаем необходимым дать качественную характеристику дыхания здоровых растений изучаемых видов пшеницы (табл. 1).

Интенсивность дыхания здоровых листьев восприимчивого вида выше, чем устойчивого; эта закономерность наблюдается во все сроки взятия проб.

Под действием ингибиторов гликолиза фторида и монойодацетата нам не удалось обнаружить существенного отличия доли участия данного пути в общем процессе дыхания изучаемых видов. Азид калия — инактиватор ряда металлосодержащих оксидаз — оказал неодинаковое действие на дыхание здоровых проростков: незначительно стимулировал поглощение кислорода у восприимчивого вида и снижал интенсивность дыхания у растений устойчивого. Различное влияние азида установлено в работе и с другими тканями (16). Обнаружение ингибирующего действия азида у растений устойчивого вида, по-видимому, указывает на участие металлосодержащих оксидаз в завершающих этапах дыхательного процесса.

Установлена различная чувствительность дыхания растений пшениц к малонату: у восприимчивого вида ингибируется 66% дыхания, а устойчивого — 50%. Очевидно, эти результаты можно рассматривать таким образом, что цикл Кребса имеет большее значение в дыхании здоровых проростков восприимчивого вида.

Заражение растений уредоспорами гриба усиливает интенсивность дыхания растений обоих видов (табл. 2). Эта закономерность наблюдается на всех стадиях развития болезни, причем возрастание уровня дыхания при поражении восприимчивого вида выше, чем устойчивого. Наибольшая разница в интенсивности дыхания обнаруживается на четвертый день после инфекции, когда проявляются первые симптомы заболевания. По мере развития гриба происходит постепенное уменьшение этой разницы.

Количественное различие в дыхании здоровых и больных грибом растений, как известно,— всеобщая закономерность, характерная при заражении как факультативными, так и облигатными паразитами. Для ре-

Таблица 2

Интенсивность дыхания здоровых и пораженных бурой ржавчиной растений пшеницы на разных стадиях поражения *
(Qo_2 на 10 мг сухого веса в час)

Вид пшеницы	Состояние растений	Интенсивность дыхания (прошло дней от начала инфекции)			Отношение дыхания больных к здоровым растениям (% на соответствующий день)		
		4	7	11	4	7	11
Tr. persicum	здоровые	109,5	102,3	153,8	100	100	100
	больные	205,4	182,2	192,7	187	178	125
Tr. timopheevi	здоровые	85,6	96,8	133,7	100	100	100
	больные	139,9	145,8	140,2	163	150	105

* Дыхание в фосфатном буфере.

шения вопроса о природе иммунитета к тем или иным заболеваниям наибольшее значение приобретает изучение тех качественных сдвигов и перестроек в нормальном процессе обмена, которые вызываются при поражении. Используя для этого дыхательные ингибиторы, мы блокировали различные ферментные системы, что дало возможность судить о качественных сдвигах в дыхании. Данные опытов с ингибиторами гликолиза приведены в таблице 3.

Уже при первых признаках поражения растений ржавчиной, через четыре дня после инфекции, начальные этапы дыхания восприимчивого и устойчивого видов разнятся. Путь дыхания, не чувствительный к фториду, условно принимается за апотомический. Сравнив между собой величины подавления фторидом дыхания у обоих видов, можно предположить, что реакция растения-хозяина, характеризующаяся видимым проявлением симптомов заболевания, связана с преобладанием гликолитического пути распада глюкозы в дыхании растений восприимчивого вида (подавление на 80%); в дыхательном процессе растений устойчивого вида наибольшее значение приобретает гексозомонофосфатный цикл. Выше уже говорилось о том, что растения устойчивого вида (Tr. timopheevi) в условиях наших опытов обнаружили типичную реакцию сверхчувствительности. По-видимому, активирование апотомического пути дыхания зараженных растений устойчивого вида может быть определенным образом связано с образованием химических фенольных барьеров, способствующих быстрой локализации инфекции, что внешне выражается в формировании многочисленных некрозов на поверхности листа.

На седьмой и одиннадцатый день после инфекции наблюдается повышение чувствительности дыхания больных растений устойчивого вида к фториду (ингибирование увеличивается на 50—78%), что может свидетельствовать о возрастающей роли гликолиза. Для растений неиммунного вида обнаруживается противоположная закономерность: по мере дальнейшего развития гриба, сопровождающегося переходом к споруляции, происходит сдвиг в начальных этапах дыхания в пользу апотомиче-

Таблица 3

Влияние ингибиторов гликолиза на дыхание пшеницы, пораженной бурой ржавчиной

Вводимое соединение, концентрация его	Вид пшеницы	Состояние растений	Интенсивность дыхания (Qo_2 на 10 мг сух. вещ. в час; прошло дней от начала инфекции)		
			4	7	11
Фосфатный буфер, pH 7,0	Tr. persicum	здоровые	109,5	102,3	153,8
		больные	205,4	182,2	192,7
	Tr. timopheevi	здоровые	85,6	96,8	133,7
		больные	139,9	145,8	140,2
Фторид натрия, $6 \cdot 10^{-2}$ M	Tr. persicum	здоровые	33,6	36,6	34,8
		больные	40,1	70,4	81,8
	Tr. timopheevi	здоровые	20,5	33,9	41,6
		больные	69,6	41,0	31,4
Монойодацетат, $1 \cdot 10^{-2}$ M	Tr. persicum	здоровые	42,7	51,2	79,3
		больные	59,0	72,2	76,8
	Tr. timopheevi	здоровые	38,8	51,0	36,6
		больные	50,8	37,8	35,7

ского пути. Подобные указания встречаются в работах Дейли (8), Фаркаша (13), которые также наблюдали активирование пентозофосфатного цикла дыхания в период активной споруляции паразита.

В таблице 3 представлены результаты опытов, проведенных с другим ингибитором гликолиза — монойодацетатом. Заражение растений ржавчиной вызывает незначительное повышение чувствительности их дыхания к этому яду на всех стадиях развития гриба. Исключение составляет пшеница устойчивого вида, когда на седьмой день после инфекции, к моменту образования очень хорошо заметных некрозов, у нее наблюдается наибольшая разница в ингибировании дыхания больных и здоровых растений (около 74%).

Неодинаковый характер реагирования окислительных систем растений на действие обоих ядов, вероятно, объясняется их неспецифичностью, особенно монойодацетата. Последний, являясь тиоловым реагентом, может взаимодействовать с рядом SH-соединений, например, с некоторыми дегидрогеназами не только гликолиза, но и цикла Кребса.

Результаты опытов с малоновой кислотой представлены в таблице 4.

Под действием малоната у растений восприимчивого вида при внедрении паразита происходят резкие сдвиги в характере дыхания. На первых стадиях заболевания реакция дыхания здоровых и больных проростков этого вида одна и та же, но по мере развития гриба дыхание пораженных образцов становится более «малонатрезистентным». На седьмой и одиннадцатый день после инфекции, когда гриб начинает размножаться, дыхание растений восприимчивого вида подавляется лишь на 20—30%. Значительное снижение чувствительности дыхания к малонату у восприимчивого вида, вероятно, указывает на уменьшение роли цикла трикарбоновых кислот в их дыхании в период активного развития ржавчины.

Явление «малонатрезистентного» дыхания наблюдали Фаркаш и Кирай (10, 11) при изучении дыхания пшеницы, пораженной стеблевой ржавчиной. Повышенное содержание некоторых органических кислот в растениях пшеницы, пораженных Puccinia graminis (9, 17, 21), очевидно, вызвано пониженной чувствительностью дыхания их к действию таких конкурентных ингибиторов, как малоновая кислота. Можно также предположить, что внедрившийся паразит вызывает изменение в дальнейшем ходе дыхательного процесса, что цикл Кребса, которому принадлежит большая роль в дыхании здоровых тканей, под влиянием инфекции при-

Таблица 4

Влияние малоната на дыхание пшеницы, пораженной бурой ржавчиной

Вводимое соединение, концентрация его	Вид пшеницы	Состояние растений	Интенсивность дыхания (Q_{O_2} на 10 мг сух. веса, час; прошло дней от начала инфекции) 4	7	11
Фосфатный буфер, pH—7,0	Tr. persicum	здоровые	109,5	102,3	153,8
		больные	205,4	182,2	192,7
	Tr timopheevi	здоровые	85,6	96,8	133,7
		больные	139,9	145,8	140,2
Малонат, $1 \cdot 10^{-2}$M, pH—4,5	Tr. persicum	здоровые	37,5	51,7	75,8
		больные	62,6	143,3	129,7
	Tr. timopheevi	здоровые	43,2	49,5	38,8
		больные	61,2	56,8	56,8

Таблица 5

Влияние азида калия на дыхание пшеницы, пораженной бурой ржавчиной

Вводимое соединение, концентрация	Вид пшеницы	Состояние растений	Интенсивность дыхания (Q_{O_2} на 10 мг сух. веса в час; прошло дней от начала инфекции)		
			4	7	11
Фосфатный буфер, pH — 7,0	Tr. persicum	здоровые	109,5	102,3	153,8
		больные	205,4	182,2	192,7
	Tr. timopheevi	здоровые	85,6	96,8	133,7
		больные	139,9	145,8	140,2
Азид калия, $5 \cdot 10^{-3}$ M	Tr. persicum	здоровые	111,3	105,4	162,2
		больные	161,7	143,3	153,3
	Tr. timopheevi	здоровые	76,9	67,2	115,1
		больные	118,2	118,0	114,9

обретает меньшее значение. Возникновение «малонатрезистентного» дыхания можно объяснить и тем, что малоновая кислота, как и целый ряд других карбоновых кислот, способна образовывать комплекс малонил-коэнзима, участвуя таким образом в процессах обмена.

Дыхание растений устойчивого вида, как показывают данные таблицы 4, под влиянием инфекции становится более чувствительным к действию малоната. Во все дни после инфекции дыхание пораженных растений подавляется малонатом примерно на 60%. Внедрение гриба почти не вызывает никаких изменений в участии цикла трикарбоновых кислот в общем дыхательном процессе.

При изучении путей дыхания пораженных ржавчиной растений использовался один из ингибиторов металлосодержащих оксидаз — азид калия. Результаты этой серии опытов представлены в таблице 5.

В литературе есть указания на незначительное активирование цитохромоксидазы при поражении растений стеблевой ржавчиной (15). Наблюдаемое в наших опытах подавление интенсивности дыхания азидом, вероятно, свидетельствует о небольшом возрастании активности металлосодержащих оксидаз при внедрении паразита.

Выводы

Уровень дыхания здоровых листьев устойчивого и восприимчивого видов пшениц различен — у второго выше. Обнаружен неодинаковый характер реагирования дыхательных систем на действие азида и малоната. В опытах с ингибиторами гликолиза не удалось установить существенных различий в доле участия начальных путей в общем дыхательном процессе.

Окислительные системы растений изучаемых видов неодинаково реагируют на заражение ржавчиной: интенсивность дыхания восприимчивого вида выше на всех стадиях развития болезни.

При заболевании ржавчиной увеличивается доля пентозофосфатного пути в дыхании к периоду активной споруляции паразита у восприимчивого вида и возрастание доли гликолитического пути — у растений устойчивого. Внедрение гриба вызывает повышение устойчивости дыхания к малонату у Tr. persicum и незначительное возрастание чувствительности к нему Tr. timopheevi. Инфекция не вызывает существенных изменений в участии цикла Кребса в дыхании листьев устойчивого вида. В опытах с азидом установлено небольшое активирование металлосодержащих оксидаз под влиянием заражения растений обоих видов. Актив-

ность завершающих оксидаз в дыхании после заражения пшеницы ржавчиной не изменилась.

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SUMMARY

The intensity of respiration in healthy leaves in rust-resistant and rust-susceptible wheat species is different: it is higher in susceptible plants. The experiments with inhibitors showed that they have different respiration modifications of the common respiratory process.

ZUSAMMENFASSUNG

Die Atmungsintensität gesunder Blätter bei rostresistenten und rostanfälligen Weizenformen ist verschieden, u. z. höher bei den letztgenannten. Im Experiment mit Inhibitoren wurde festgestellt, daβ die Atmungswege im allgemeinen Atmungsprozess bei ihnen verschieden sind.

RESUME

L'intensité de respiration des feuilles saines des espèces des blés résistantes et sensibles à la rouille est différente. Celle de l'espèce sensible est supérieure. Les essais avec les inhibiteurs ont permis de constater que les deux espèces présentent les différentes voies de respiration dans le procesus général respiratoire.

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Purification and Properties of Tobacco Ringspot Virus and Two RNA-Deficient Components[1]

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Accepted December 2, 1964

Partially purified tobacco ringspot virus exhibited three schlieren peaks in the analytical ultracentrifuge. Centrifugation on a sucrose density gradient resulted in three zones having sedimentation coefficients of 53, 94, and 128 S at the top, middle, and bottom, respectively. Infectivity was associated with the bottom zone. Particles in all three zones were serologically identical. Negatively stained particles examined in the electron microscope were the same size and differed only in nucleic acid content. The ultraviolet absorption spectra of the three zones showed a transition from nucleoprotein in the bottom to protein in the top component. The molecular weight of the bottom component was calculated as 4.3 million, of which 42% was RNA. Amino acid analysis indicated that each subunit contained at least 177 residues, not including tryptophan and cysteine, and weighed a minimum of 19,300.

INTRODUCTION

Several instances are known in which spherical plant viruses exhibit more than one schlieren peak when purified preparations are examined in an analytical ultracentrifuge. For example, Mazzone *et al.* (1962) found that purified preparations of squash mosaic virus contained three particles with sedimentation coefficients of 57, 95, and 118 S. They concluded that the protein coats of the three particles were similar or identical and that the particles differed mainly in their content of RNA. Confirmation of the loss of RNA in particles of turnip yellow mosaic virus was demonstrated by electron microscopy using the negative staining technique, when the term "ghost" particle was applied to the empty protein shell (Brenner and Horne, 1959). Tobacco ringspot virus was shown by Steere (1956) to belong to that group of viruses that had more than one particle associated with purified preparations. He found that the bulk of his material had a sedimentation coefficient of 116 S while some 5% of it had a lower sedimentation coefficient (89 S). Infectivity was confined to the full (116 S) particles.

The present investigation arose from attempts to purify tobacco ringspot virus for serological purposes. In the purification procedures that were used, partially purified material consistently showed three peaks when examined in the analytical ultracentrifuge using the schlieren optics. Material comprising these three peaks were separated from one another on a sucrose gradient, and their properties are the subject of this paper.

MATERIALS AND METHODS

The virus inoculum was obtained from Dr. C. D. McKeen, Harrow, Ontario. The cucumber *Cucumis sativus* L. var. National Pickling was found to be a suitable host for producing the virus in bulk. Seedlings were inoculated at the cotyledon stage and harvested about 10 days later, when the cotyledons showed chlorotic local lesions and the true leaves showed systemic chlorosis. All

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treatments for the purification of the virus were carried out at or near 5°C. Low speed centrifugations were done in the Servall RC-2 refrigerated centrifuge and high speed centrifugations in the Spinco model L preparative centrifuge, using either the 30, 40, or SW 25 rotor. The degree of purification and relative loss at each stage of purification was checked in the Spinco model E analytical ultracentrifuge using the schlieren optics (Markham, 1962).

The purification procedure consisted of the following steps:

1. Infected tissue was ground in a meat grinder using a buffer of 0.5 *M* boric acid (containing 0.1% sodium thioglycolate and 0.1% diethyldithiocarbamate) adjusted to pH 6.8 with NaOH in a proportion of 1 kg tissue to 1 l buffer.

2. Ground material was homogenized in a blendor for 2–3 minutes and squeezed through cheesecloth.

3. The sap was frozen overnight, thawed, and centrifuged at 10,000 *g* for 30 minutes.

4. The supernatant was dialyzed for 16–24 hours against 5 volumes of ammonium sulfate, 200 g/l.

5. The dialyzate was centrifuged at 10,000 *g* for 30 minutes and the clarified dialyzate was pelleted in the no. 30 rotor at 28,000 rpm for 120 minutes.

6. The pellets were suspended in 0.1 *M* ethylenediamine tetraacetate (EDTA) pH 7.0, and the suspension was clarified in the no. 40 rotor at 15,000 rpm for 10 minutes.

7. The clarified virus was recentrifuged in the no. 40 rotor at 38,000 rpm for 60 minutes, and the pellets were resuspended in EDTA and clarified as in step 6.

8. The purified virus was layered onto an unbuffered 5–30% sucrose gradient and centrifuged in the SW 25 rotor at 23,000 rpm for 150 minutes. The three zones that resulted, designated top (T), middle (M), and bottom (B), were drained off separately with a needle inserted through the bottom of the tube.

9. The products of the separate zones were dialyzed against 0.01 *M* Tris acetate buffer, pH 7.0, containing 0.1 *N* sodium chloride.

For infectivity tests, each of the zonal components (T, M, and B) was adjusted to give an absorption of 0.400 at 280 mμ and diluted 1:10 for inoculation. Local-lesion assays were made on plants of *Nicotiana tabacum* L. var. Xanthi. The half-leaf method was used to compare inocula.

Antisera of the three components were produced in rabbits by intramuscular injection of 0.2–0.5 mg of each component, either as a single injection or as two injections 14 days apart. For injection the virus in physiological saline was emulsified in equal volumes of Freund's incomplete adjuvant. Antisera were obtained 2 weeks or more after the initial injection and were preserved by freezing.

The dilution end point of each antiserum was determined first with the homologous component and then with each of the two nonhomologous components.

Three dilutions of antisera of each component were cross-absorbed with an equal volume containing 30 μg of nonhomologous component per milliliter. All other aspects of cross-absorption and complement fixation were the same as those used in tests with virus strains (Wright, 1963). Results were expressed as a component specific antibody index for each antiserum absorbed to the 85% level or higher. The index was derived by subtracting from the absorption level the mean per cent hemolysis occurring in tubes containing homologous antigen.

Sedimentation coefficients were measured in the Spinco model E analytical ultracentrifuge using UV optics. Virus concentrations were chosen in such a way that the optical density of the solutions at 260 mμ was of the order of 1.0. The photographs were scanned with the Joyce Automatic Recording Microdensitometer, and the sedimentation coefficients were calculated for standard conditions (20°C in water).

The diffusion coefficient was measured in the Spinco model H electrophoresis-diffusion apparatus by means of the integral fringe method (Schachman, 1957). Measurements were made at 4° in 0.1 *M* sodium chloride at intervals during 75 hours and were converted to diffusion coefficients under standard conditions (20° in water).

The nucleic acid and protein composition of the virus was determined as follows: A solution containing 3–5 mg of purified virus

was centrifuged at 38,000 rpm for 1 hour. The pellet was suspended in a drop of distilled water, and the nucleic acid was split from the suspended virus by adding 2 ml of 1.0 *N* HCl to it and keeping it at room temperature for 24 hours. The denatured protein was pelleted by centrifugation at 15,000 rpm for 10 minutes and washed three times with 0.8 ml of 1.0 *N* HCl. The supernatants were collected and made up to 5 ml with 1.0 *N* HCl. A measured aliquot was sealed in a test tube, heated for 1 hour in boiling water, and evaporated to dryness at 40° in an air current; the residue was transferred quantitatively in 0.1 *N* HCl on a Whatman no. 40 filter paper strip. The nucleotides were separated chromatographically in isopropanol, hydrochloric acid, and water, and determined according to Smith and Markham (1950). The protein pellet was hydrolyzed in 6 *N* HCl in an evacuated sealed tube for 24 hours and an aliquot was analyzed with a Spinco Amino Acid Analyzer with 60-cm columns. The protein concentration was obtained by adding up the individual amino acids, and the virus concentration was calculated as the sum of the nucleic acid and protein concentrations. Phosphorus analyses were made according to Michelson (1957). The nucleic acid content was calculated from an average nucleotide residue of 321, giving a phosphorus content of 9.66%.

RESULTS

Purification

The preparative procedures outlined in the previous section did not incur serious losses of virus material as ascertained from the areas under the peaks in the schlieren photographs. A typical preparation, prior to final concentration on a sucrose gradient, is shown in Fig. 1a. Three peaks are evident, and such preparations consistently yielded three bands on the density gradient (Fig. 1b).

Modifications of the basic procedure were tested to determine whether any of the three peaks were artifacts resulting from a step in the purification, particularly freezing and dialysis against ammonium sulfate. These modifications demonstrated that the peaks were consistently present in pelleted material and were not induced by freezing or by dialysis against ammonium sulfate, although some of the early steps in the purification procedure could have caused the full particles to break down into noninfectious components. When material was processed unfrozen or when ammonium sulfate was omitted, three peaks still resulted but, because of the higher level of impurities, inconsistencies appeared at the density gradient stage of purification. The virus often did not separate into three distinct zones, and a large proportion of the virus material frequently pelleted on the bottom of the sucrose gradient tube.

The ammonium sulfate step in the purification procedure eliminated a schlieren peak that was present in both healthy and diseased sap and that sedimented at a rate between 70 and 80 Svedberg units. Virtually no virus was precipitated by the ammonium sulfate at the concentration used, but if the concentration was increased to 300 g/l most of the virus was precipitated along with normal plant material. The ammonium sulfate need not have been added to the sap through dialysis. Granular ammonium sulfate slowly added to the clarified sap at the rate of 180 g/l was equally effective. The advantage of dialyzing against a solution of ammonium sulfate was that localized salt concentrations were avoided and dialysis reduced the volume of clarified sap by nearly a third, hence partially concentrating the virus.

The butanol–chloroform procedure used by Steere (1956) for purifying tobacco ringspot gave yields of partially purified virus comparable with those obtained by the method described here. Material processed by the butanol-chloroform procedure also showed three peaks in the schlieren optics in the relative proportion of those shown in Fig. 1a.

Stabilizing the purified zones that were drained from the density gradient tubes was difficult. Sucrose was normally removed by dialysis against a large volume of 0.01 *M* Tris buffer at pH 7.0. Dilute preparations, containing 1–2 mg/ml, formed a visible precipitate in the dialysis bag or upon storage at 4°. When the bottom zone preparation was rerun on a density gradient, three zones

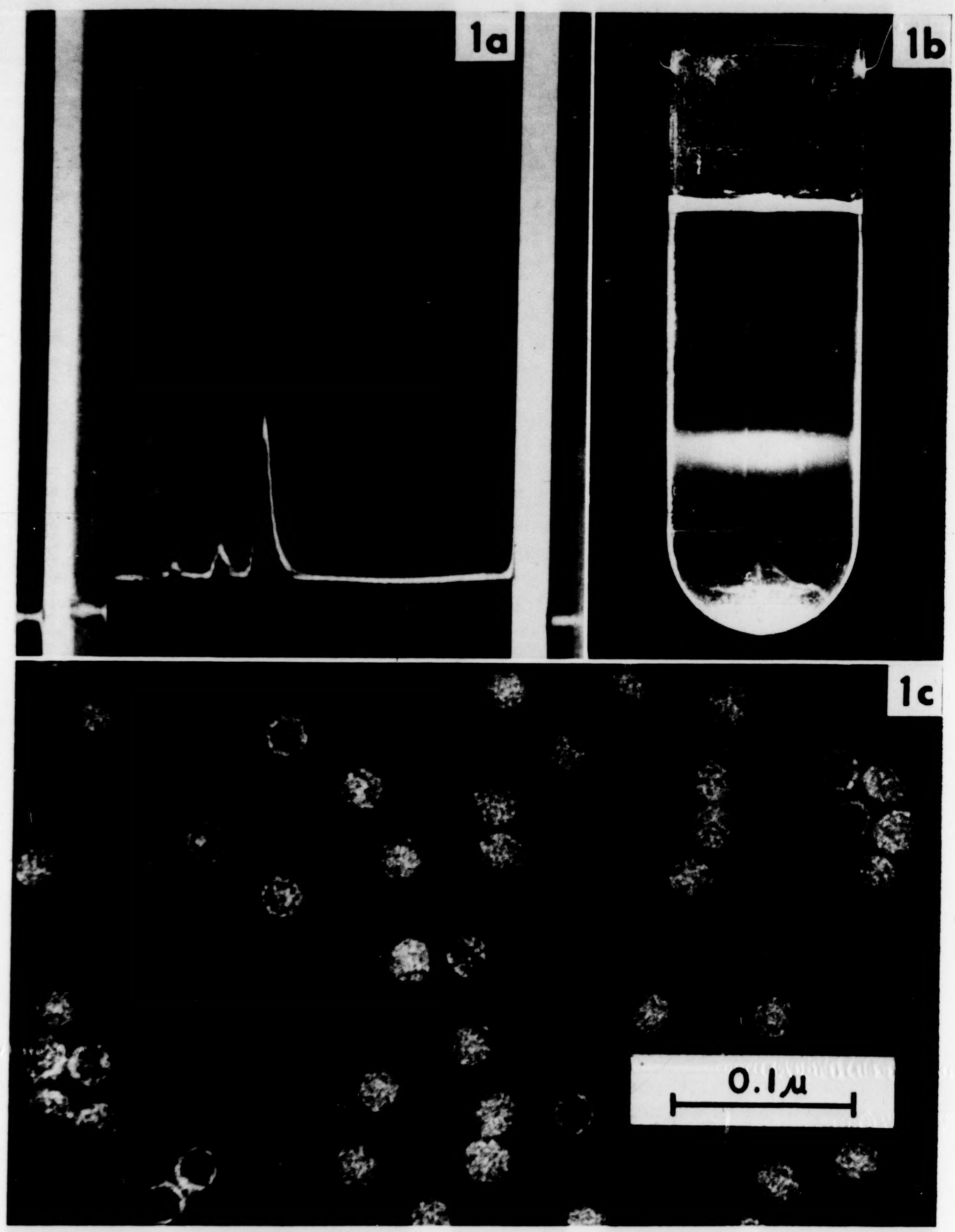

FIG. 1. (a) Analytical ultracentrifuge photograph of partially purified tobacco ringspot virus showing relative proportion of the three components. Photo taken with schlieren optics 10 minutes after reaching speed of 35,600 rpm. (b) Density gradient tube showing the three zones that result from layering material similar to that photographed in Fig. 1a, and centrifuging 150 minutes at 23,000 rpm. (c) Electron micrograph of particles drained from bottom zone of density gradient tube.

again resulted, indicating that a proportion of the bottom zone material had degraded into lighter particles. It was later found that high concentrations of virus could be stabilized by including 0.1 *N* sodium chloride in the buffer. Bottom zone material stored under such conditions yielded a single zone when rerun on a density gradient.

Sedimentation Coefficients

The sedimentation coefficients of the isolated components at concentrations corresponding to 1 optical density unit at 260 mμ were S_{20w} = 128, 94, and 53 S for the bottom, middle, and top component, respectively. Purified zones from four separate preparations were measured, and the results were in good agreement.

Infectivity

The results of a typical infectivity test are summarized in Table 1, showing that 97% of the infectivity was in the bottom component while the remaining 3% was confined to the middle component. Other infectivity tests gave similar results so long as the three zones were slowly drained from the bottom of the tube. When the zones were sucked off through a needle inserted from the top of the tube the liquid was disturbed and the middle zone accounted for up to 10% of the infectivity.

Serology

The dilution end points of all antisera obtained at least 2 weeks after the initial injections were 1:640; from later bleedings following a second injection they were 1:1280 or 1:2560. The same end points were found in tests with homologous and nonhomologous antigens. This result, indicative of serological identity of the three components, was confirmed by tests with cross-absorbed antisera. In no case was the component-specific antibody index above zero (Table 2).

TABLE 1

INFECTIVITY OF THE TOP (T), MIDDLE (M), AND BOTTOM (B) COMPONENTS OF TOBACCO RINGSPOT VIRUS ON HALF-LEAVES OF TOBACCO

Local lesions per half-leaf[a]					
TM		MB		BT	
0	2	10	180	210	1
0	3	8	205	255	0
0	1	11	178	260	0

[a] Each component was compared with each of the other two components, and the test was replicated three times. Inoculum concentration was approximately 5 μg per millilitre.

TABLE 2

COMPLEMENT FIXATION TESTING OF CROSS-ABSORBED ANTISERA OF THREE COMPONENTS (T, M, AND B) OF TOBACCO RINGSPOT VIRUS FOR COMPONENT-SPECIFIC SEROLOGICAL ACTIVITY

Antiserum	Absorbed with	Number of tests	Mean index[a] ± SE	Significance by *t* test (5% level)
T	M	4	6.0 (±3.5)	Nil
T	B	3	3.7 (±2.2)	Nil
M	T	4	0.5 (±1.5)	Nil
M	B	4	−1.5 (±1.0)	Nil
B	T	3	−0.7 (±0.6)	Nil
B	M	3	1.3 (±1.3)	Nil

[a] Of component-specific antibody.

Electron Microscopy

Figure 1c shows an electron micrograph of a preparation from the bottom component negatively stained with 1% phosphotungstic acid. The particle diameter was found to be approximately 25 mμ, which is consistent with earlier work (Corbett and Roberts, 1962; Steere, 1956). Electron micrographs of the bottom component always included a few empty particles, and micrographs of the top component occasionally included a few full ones, but the particle diameters of the three components were identical. The resolution of the photographs was not adequate to make a clear distinction between the middle and top components, but in many particles from the middle zone the hole appeared distinctly smaller than the hole in the top component.

Ultraviolet Absorption

The ultraviolet spectrum of the three components is shown in Fig. 2, demonstrating

the transition from a nucleoprotein (bottom component) to a protein (top component). The maximum absorption of the top component has shifted from 260 mμ to approximately 275 mμ, consistent with the general shape of a protein spectrum. The 260 mμ reading is still probably too high since the 280:260 ratio in proteins is usually in the order of 1.5–2.0 whereas in this case it is only 1.07. However, because of the very strong absorption of RNA at 260 mμ, about 1% contamination with RNA would account for this ratio. Slight color contaminations in the preparation would have a similar effect.

Molecular Weight

The diffusion coefficient of the bottom component gave a value of $D_{20w} = 2.0 \times 10^{-7}$ cm/sec. The molecular weight (M) was calculated from the following expression:

$$M = \frac{RT \times S_{20w}}{(1 - \bar{V}\rho) \times D_{20w}}$$

(where R = gas constant, T = absolute temperature, $\bar{V}$ = partial specific gravity, ρ = density of water at 20°). The result

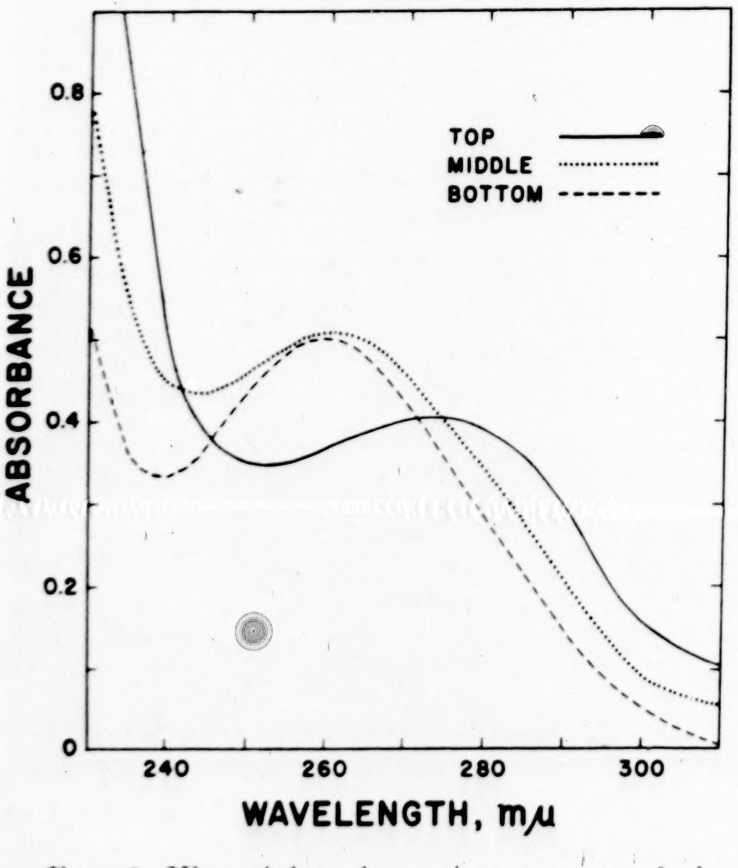

FIG. 2. Ultraviolet absorption spectra of the top, middle, and bottom components of tobacco ringspot virus. The approximate concentrations were 0.064, 0.052, and 0.039 mg/ml of the top, middle, and bottom components, respectively.

TABLE 3

AMINO ACID ANALYSIS IN DUPLICATE OF A SAMPLE OF TOBACCO RINGSPOT VIRUS PROTEIN

Amino acid	Grams of amino acid per 100 g virus protein		Integral number of residues[a]	Minimum molecular weight ($\times 10^3$)[b]	
	Sample 1	Sample 2		Sample 1	Sample 2
Lysine	6.1	6.2	9	18.9	18.6
Histidine	4.3	4.3	6	19.1	19.1
Arginine	6.7	6.7	8	18.6	18.6
Aspartic acid	9.9	10.2	17	19.8	19.2
Threonine	6.7	6.8	13	19.6	19.3
Serine	6.2	6.4	14	19.7	19.0
Glutamic acid	9.3	9.4	14	19.4	19.2
Proline	5.5	5.6	11	19.4	19.1
Glycine	4.6	4.7	15	19.3	18.8
Alanine	5.5	5.6	15	19.4	19.0
Valine	5.5	5.6	11	19.8	19.5
Methionine	2.1	2.0	3	18.7	19.7
Isoleucine	6.6	6.3	11	18.9	19.8
Leucine	8.5	8.1	14	18.6	19.6
Tyrosine	5.1	4.7	6	19.2	20.8
Phenylalanine	7.4	7.4	10	19.9	19.9
Mean	—	—	—	19.3	19.3

[a] Calculated for an assumed molecular weight in the order of 20,000.

[b] Calculated from the integral number of residues tabulated in adjacent column.

obtained was 4.3×10^6 using 0.64 for $\bar{V}$ as calculated from the RNA content.

RNA and Protein Analyses

The RNA content of the bottom component by direct determination and phosphorus analyses was 41.9% and 42.1%, respectively. The nucleotide composition in mole per cent (average of four determinations) were G = 24.7, A = 23.1, C = 22.4, and U = 29.8. The sedimentation coefficient of RNA prepared by treating the purified bottom component with phenol was 28 S when a solution containing 1.0 optical density unit was measured in the UV optics.

Table 3 shows the amino acid composition of the viral protein of a duplicate analysis. The second column gives grams of amino acid residue per 100 g protein. If the number

of amino acid residues was calculated for a minimum molecular weight of the order of 20,000, each amino acid was close to an integral number, which is shown in column 3. These result in a total of 177 amino acid residues, not counting tryptophan and cysteine, which were not determined. Column 4 is the molecular weight calculated from the integral number of residues given in column 3.

DISCUSSION

The sedimentation coefficient of the heavy component of 128 S is in reasonable agreement with the value of 116 S given by Steere (1956). The lower value obtained by Steere may have resulted from the fact that his measurements were made at high virus concentrations with the schlieren optics. The 89 S component reported by Steere obviously corresponded to our middle component of 93 S. Steere did not report a third component corresponding to our 53 S component, possibly because his strain of tobacco ringspot virus was more stable than the one used in this study.

The ratio of nucleotide composition is in good agreement with a previous report (Kaper and Steere, 1959). The RNA content of 42% is somewhat higher than the value of 34.4% reported by Steere (1956) and is in closer agreement with an earlier report of 40% by Stanley (1939). If one assumes that the molecular weight of RNA in this virus is 2×10^6, as indicated by a sedimentation coefficient of 28 S, and taking our molecular weight of the virus (4.3×10^6), then the calculated RNA content would be 46.5%. The discrepancy between calculated and determined RNA content may mean that the molecular weight is somewhat greater than 4.3 million, resulting from an error in the diffusion coefficient as discussed below.

The ratio of the sedimentation coefficients of the bottom and top components is 2.42. It has been shown that this ratio depends upon the RNA content only (Reichmann, 1965), and from the simplified equation the RNA content is calculated as 41.4%, which is in good agreement with the determined value of 42%. The ratio of the sedimentation coefficients of the middle and bottom components is 1.77, from which an RNA content of 27.8% can be calculated for the middle component. This means that particles forming the middle zone have lost approximately one-third of their nucleic acid content.

The diffusion coefficient of 2.0×10^{-7} found by means of the integral fringe method may be somewhat high. With this method the most reliable data are obtained after long diffusion times. In our case the longest diffusion time was 75 hours. Also, the purified preparation had been stored for a day in the absence of 0.1 *N* sodium chloride and as a consequence had degraded somewhat. This degradation may have influenced the diffusion coefficient towards a higher value, and the molecular weight derived from the latter towards a lower value.

The amino acid composition of this virus as shown in Table 3 does not display any unusual features. The actual value of the molecular weight which fits an integral value for every residue is given in column 4. The average molecular weight of the subunit was found in this way to be 19,300, the maximum was 20,800 for one determination of tyrosine, and the minimum 18,600 for arginine. Since tryptophan, cysteine, and ammonia were not determined, the value for the molecular weight of the subunit would tend to be low. This value corresponds to the minimum molecular weight and the actual molecular weight could be a multiple of this figure.

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Radiation Effects on Polyoma Virus

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Polyoma T and its host mouse embryo cells were irradiated with Co^{60} radiation both alone and as infectious complexes. The virus alone is very radiation resistant and in the presence of radical scavengers has a Do of 4.09×10^5 rads. In contrast the cellular capacity has a Do of 5440 rads. Infectious complexes of virus infected cells have a Do after infection of 7800 rads. This value increases progressively during the eclipse period and after 19 hr. approaches the value for free virus. These differences are interpreted as changes in the "capacity" of the host cell during the eclipse period of virus maturation.

INTRODUCTION

The properties of polyoma virus have been widely studied particularly because of the different responses this virus elicits in different host cells. In some systems a virus–cell interaction leading to the production of malignant cells (Vogt and Dulbecco, 1960) predominates while in others, notably the mouse embryo cell *in vitro,* the usual reaction is viral multiplication leading to a cytopathic effect.

Very little is known about the stages of development of polyoma virus in infected mouse cells except that it has been shown that synthesis of viral DNA occurs in the cell nucleus just prior to virus maturation (Williams and Sheinin, 1961). One approach to this problem was suggested by the experiments of Luria and Latarjet (1947) which utilized radiobiological techniques to study bacteriophage development, and we have attempted to employ this technique to study polyoma virus development. The experiment consists of determining a survival curve of the ability of infected cells to produce virus at various times during the virus latent period. The rationale behind the experiment is as follows: The radiation-sensitive site within the complex is presumed to be the viral genome rather than some function of the cell (i.e., cellular "capacity"). Thus as virus replication proceeds in the cell, the number of genomes or sensitive sites in each complex should increase. Since only one genome need survive radiation treatment to give an infectious complex, the radiation survival curves should develop significant shoulders as the genome multiplies before irradiation. Thus measurement of a series of these curves at intervals throughout the latent period should provide data bearing on virus replication.

The partial success of this approach in the study of the bacteriophage has led to the application of these techniques to systems involving various animal viruses (Powell, 1959; Franklin, 1958; Kaplan, 1962; Rubin and Temin, 1959; Dulbecco and Vogt, 1955; Friesen *et al.,* 1963).

In this paper we report on studies using Co^{60} radiation and the Luria–Latarjet technique with the polyoma T–mouse embryo cell system. As part of this study the sensitivity to Co^{60} radiation of polyoma T (McCulloch *et al.,* 1959) virus and of the capacity[1] of mouse embryo cells were also investigated. The results indicate that changes of

[1] Capacity is defined as the ability of an irradiated cell to produce virus when infected after irradiation.

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Interactions of Turnip Yellow Mosaic Virus with Quaternary Ammonium Salts

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In the presence of quaternary ammonium salts of the general structure $C_nH_{2n+1}N \cdot Me_3Br$, rapid degradation of turnip yellow mosaic virus takes place at pH 9 and 20°C. In contrast, nucleic acid free protein shells of the virus are much more stable under the same conditions.

The way in which the rate of the reaction depends on the pH and on the temperature of the reaction mixture and on the concentration and the structure of the quaternary ammonium salts used has been investigated. A large increase of the rate was found when $n = 6$, while only insignificant increases could be detected when $n < 6$. The degradation by means of the quaternary salts has been compared with the behaviour of the virus in solutions of alkali salts.

Interaction of the virus with sodium dodecyl sulphate, spermine, a primary and a tertiary amine, have been studied; as with the quaternary ammonium salts with $n < 6$ no significant increases of the rate were found.

As to the mechanism of the degradation, it is suggested that the quaternary ammonium ions cause conformational changes of the nucleic acid inside the virus particles, resulting ultimately in degradation of the virus. This view is based among other considerations on the sedimentation behaviour of free virus nucleic acid in solutions of quaternary ammonium salts.

INTRODUCTION

In a series of investigations in our laboratory, Kaper (1960a, b) has shown that turnip yellow mosaic virus (TYMV) can be split into the undenatured virus protein (ATC = artificial top component) and the degraded virus ribonucleic acid (TYMV RNA), by treatment of the virus with alkali salts at pH 11–12 at 30°C. At ionic strengths greater than 0.1, comparatively large amounts of ATC are produced (Kaper, 1964), whereas at lower ionic strengths or at higher pH, more protein components with lower sedimentation coefficients appear.

The nature of these reactions led us to suppose that small ions penetrated the virus particles before these lost their nucleic acid, which was subsequently degraded in the alkaline reaction mixture. In order to obtain more information about the mechanism of this degradation reaction and about the influence of the nature of the ions taking an active part in the reaction, we studied the effect of several quaternary ammonium salts in this reaction. In this way salts having different structures could be tested, the degree of dissociation remaining of the same order as that of the alkali salts.

Preliminary experiments in which quaternary ammonium ions replaced the alkali ions in the reaction mixture indicated a sharp increase in the rate of the virus degradation, in contrast to the expected possible retardation caused by steric factors. It was decided therefore to study the degradation reaction in the presence of quaternary ammonium salts, which took place at a lower pH and a lower temperature, and to examine the influence of the structure of those ions on the rate of the reaction (cf. Stols, 1964).

While the intact virus particles were labile in solutions of several of the quaternary

ammonium salts used, ATC and the natural top component (NTC) were much more stable under the same conditions. This places the degradation of TYMV by quaternary ammonium salts, together with the alkaline degradation (Kaper, 1960a, b, 1964), in quite a different group of reactions from that to which the degradation by protein reagents belongs. In our laboratory Godschalk (1964) has studied the degradation of TYMV by alkyl mercuric salts. He has obtained similar results in regard to the effect of the length of the alkyl group on the rate of virus degradation. In this case, however, the top components and the virus are equally reactive: the presence of RNA is not essential as in our case.

MATERIAL AND METHODS

Virus. TYMV was grown in Chinese cabbage (*Brassica chinensis* L., var. 'Wong Bok') in the greenhouse as described by Markham and Smith (1949).

Isolation and purification of the virus. The virus was isolated from the sap of infected plants by the alcohol–ammonium sulfate method of Markham and Smith (1949) or by the butanol–chloroform and high speed centrifugation method of Steere (1956). The virus and the accompanying top component were separated and further purified by differential centrifugation, CsCl density-gradient centrifugation (Matthews, 1960), or by zonal rate centrifugation as described by Brakke (1953, 1960). Preparation III was separated from the sap of the 1961 harvest (which had been stored frozen) and purified by differential centrifugation. Preparation VII, from the 1962 harvest, was prepared from fresh sap and purified by CsCl density-gradient centrifugation. Preparation VIII, coming from both the 1962 and the 1963 harvests, was purified from fresh plant sap, by zonal rate centrifugation in sucrose gradients.

Virus characterization. Sedimentation patterns of the virus and the top component were used to judge the purity of the preparations; sedimentation constants agreed well with values found in the literature. Absorption spectra were measured and served as a means to determine the concentration. For TYMV, $A_{1\text{cm}}^{1\%}$ (260 nm[1]) = 85 and for NTC or ATC, $A_{1\text{cm}}^{1\%}$ (274 nm) = 12.8 was used (Kaper, 1964). The RNA content of the virus was taken to be 39% (Haselkorn, 1962).

Preparation of ATC. ATC was prepared according to Kaper (1964). The product was not extensively purified, as contamination with small amounts of more or less degraded RNA did not interfere. Sedimentation patterns failed to show intact TYMV.

Preparation and characterization of TYMV RNA. TYMV RNA was prepared from TYMV-VIII in a coldroom at 4°C using phenol (Gierer and Schramm, 1956) and bentonite (Brownhill *et al.*, 1959; Fraenkel-Conrat *et al.*, 1961). After two extractions of the virus solution (containing 0.001 M magnesium acetate) with phenol, the aqueous solution was extracted 5 times with peroxide-free ether. The ether was removed by passing nitrogen through the solution. The RNA thus prepared sedimented as a 70–90% homogeneous boundary with a sedimentation coefficient of about 30 S in 0.1 M KCl, pH 6 (36 S in the presence of 0.001 M spermine). Of RNA prepared without bentonite (Hitchborn and Dunn, 1963) the base ratio was determined according to Markham *et al.* (1951). The result (G/A/C/U = 16.8/22.8/38.0/22.3) compared favourably with that of Symons *et al.* (1963) (type strain G/A/C/U = 17.2/22.4/38.3/22.1).

Quaternary ammonium salts. Most of the quaternary ammonium salts used were the bromides, prepared by allowing the corresponding alkyl bromides to react during 4–10 hours in a closed vessel at 80°–90°C with equimolar amounts of 33% (w/w) trimethyl amine in ethyl alcohol (van Senden, 1960). Dry diethyl ether was then added to the reaction mixture, and the crystalline mass was recrystallized several times from cold ethyl alcohol by adding diethyl ether. Most of the shorter-chain quaternary ammonium salts were very hygroscopic. Solutions were made in distilled water, and the concentration was determined by argentometric titration. Gas chromatographic analyses showed no more than 1.5–4% of impurities in the

[1] nm = nanometer, equivalent to millimicron.

alkyl bromides used. The solutions of the quaternary ammonium bromides sometimes did show a slight absorbance at 254 nm; however, values between 0.01 and 0.04 were usually found.

Ultracentrifugation. Reaction mixtures were examined in the Spinco analytical ultracentrifuge (Beckman Instruments, Inc.), model E, using schlieren optics as well as ultraviolet (UV) absorption optics. Pictures were taken on Perutz Spektralplatte 550 (Gelb) for the schlieren system and on Ilford Process film for the ultraviolet system. The UV pictures were scanned on the Analytrol densitometer, equipped with a microanalyzer attachment (Beckman Instruments, Inc.).

Reactions. The reactions were carried out in thermostatted glass or plexiglass vessels. Temperature was held constant within 0.05°C. The contents could be stirred mechanically or magnetically, and samples could be withdrawn or reagents added with a syringe. The reactions were carried out under a slight overpressure of nitrogen. The pH was measured with a combined glass electrode (Metrohm EA 125X), attached to a Metrohm pH-meter Titriskop E166. Standardization was done with Sörensen's standard buffer solutions. The pH could be read with a precision of 0.02 unit and was kept constant within 0.05 unit of the desired value by manually adding 0.1 *M* NaOH with an Agla micrometer syringe.

Measuring of the percentage of undegraded TYMV. This was done by taking an aliquot from the reaction mixture, stopping the reaction by lowering the pH, diluting to an absorbancy of about 0.5, and centrifuging in the Spinco E. Several pictures were taken with UV optics, and from the densitographs the relative amount of material sedimenting with about 115 S could be determined (for general methods see Svedberg and Pedersen, 1940; Schachman, 1959). Standard curves were made with mixtures of known composition of uridylic acid (with known A^{260}_{1cm}) and TYMV. Corrections were applied, if necessary, for the absorbance of reagents present.

RESULTS

Influence of the pH on the Rate of Virus Degradation

Preliminary experiments showed that no virus degradation takes place at pH 6.5 or lower. As a rule samples were brought to pH 6.0 with 0.05 *M* sodium acetate buffer before centrifuging in the Spinco E, ensuring no further reaction after the samples had been taken from the reaction mixtures. Figure 1 contains the percentage intact TYMV remaining after different intervals for three TYMV preparations at two pH values. The rate of virus degradation is much higher at pH 9 than at pH 8, the difference between the three virus preparations remaining qualitatively the same.

Influence of Temperature on the Rate of Virus Degradation

Degradation was measured at 15°, 20°, and 25° C. Generally for all other reactions 20° was chosen. The influence of temperature, shown in Fig. 2, is somewhat more than expected; it is evident that good control of temperature is important.

Influence of the Concentration of the Quaternary Ammonium Salts on the Rate of Virus Degradation

Most reactions in this investigation were carried out in the presence of 0.20 *M* of a quaternary salt without any alkali salt or buffer present. In some cases the reaction rate turned out to be very sensitive to the concentration of the quaternary ammonium salt or to the presence of alkali ions, as is shown in Fig. 3. Addition of NaBr to a quaternary salt decreases the rate of degradation of the virus considerably.

Influence of the Structure of the Quaternary Ammonium Salts on the Rate of Virus Degradation

Several quaternary ammonium salts of the type $C_nH_{2n+1}NMe_3Br$ with straight-chain alkyl groups have been used in studying the effect of the structure on the rate of virus degradation. Salts with $n > 8$ caused formation of insoluble products, thus restricting the investigation to the salts with $n \leq 8$. In Fig. 4 the results of a number of experi-

ments with a series of homologous salts are represented. The action of salts with $n \leqq 5$ did not differ significantly from the rate of degradation measured in the presence of NaBr. With $n = 6$, 7 or 8, a significant

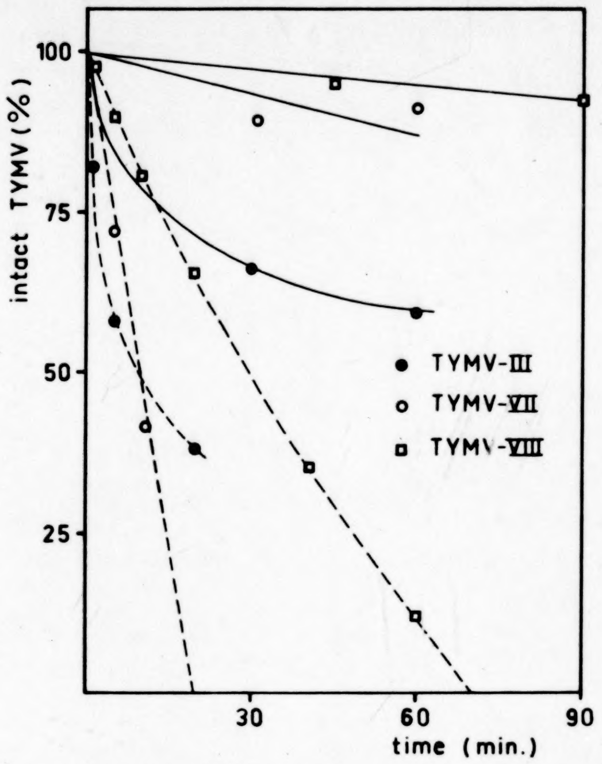

FIG. 1. Degradation of TYMV (0.14 mg/ml) in 0.20 M $C_6H_{13}NMe_3Br$ at pH 8.0 (——) and at pH 9.0 (-----) for three different virus preparations. Temperature 20°C.

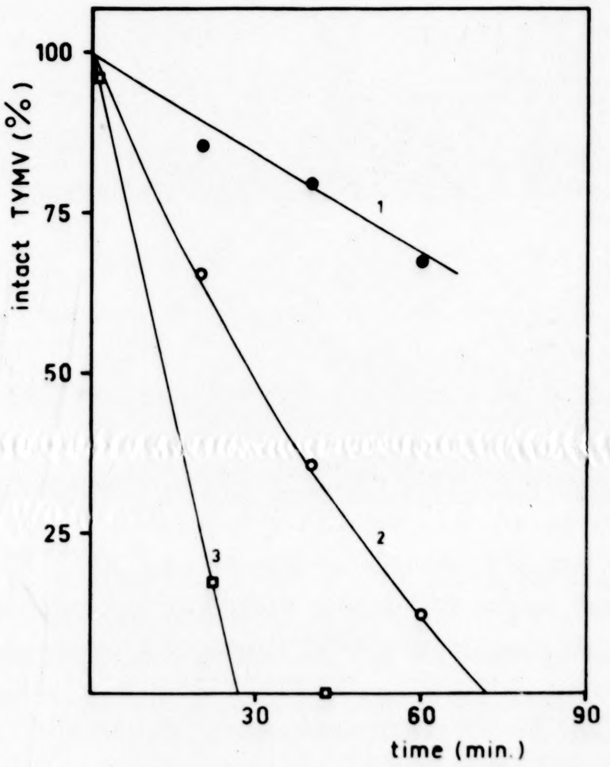

FIG. 2. Degradation of TYMV-VIII (0.14 mg/ml) in 0.20 M $C_6H_{13}NMe_3Br$ at pH 9.0, conducted at three different temperatures: Curve *1*, 15°C; *2*, 20°C; *3*, 25°C.

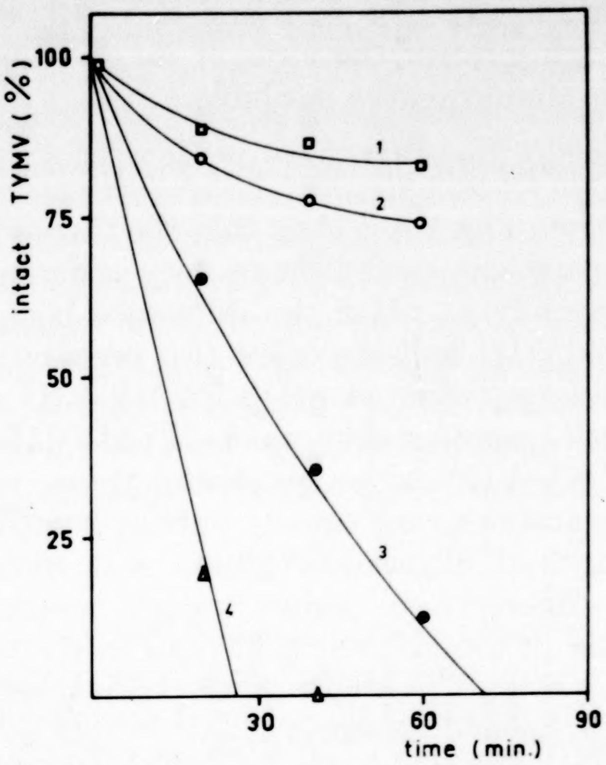

FIG. 3. Degradation of TYMV-VIII (0.14 mg/ml) in solutions of $C_6H_{13}NMe_3Br$ at pH 9.0 and 20°C. Curve *1*, 0.1 M quaternary salt + 0.1 M NaBr; *2*, 0.1 M quaternary salt; *3*, 0.2 M quaternary salt; *4*, 0.4 M quarternary salt.

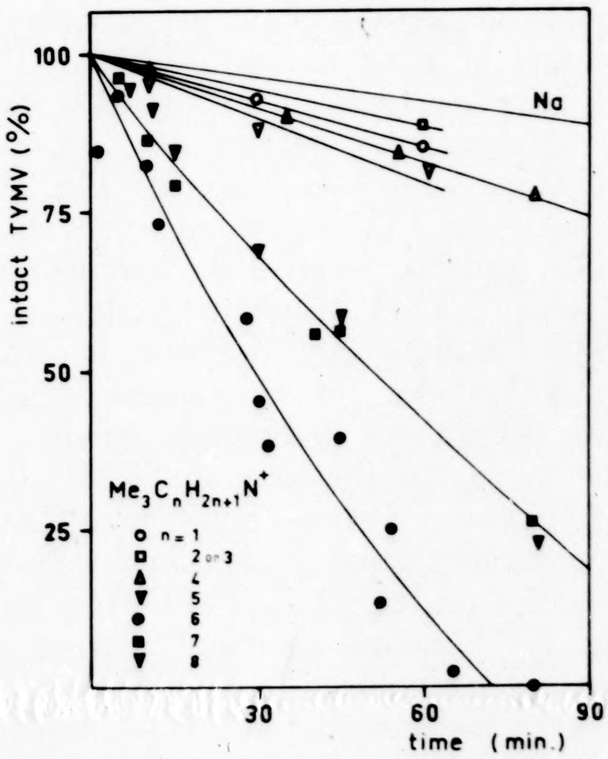

FIG. 4. Degradation of TYMV-VIII (0.14 mg/ml) in 0.2 M $C_nH_{2n+1}NMe_3Br$ at pH 9.0 and 20°C. The uppermost line marked "Na" represents the degradation of TYMV-VIII in a solution containing *only* 0.2 M NaBr.

increase of the rate of degradation was found, the rate being the highest for $n = 6$.

Some indications have been obtained that virus degradation also takes place with

salts for which $n = 9$ and $n = 10$, even though most of the virus or its decomposition products were insoluble.

Properties of Different Virus Preparations

From Fig. 1 it is clear that the three virus preparations used behave very differently. Especially at pH 8 the difference between TYMV-III and the other two preparations was large, while at pH 9 TYMV-VIII was much more stable than TYMV-III or TYMV-VII. Although these three virus preparations were equally pure as judged by analytical ultracentrifugation and whereas no difference in sedimentation properties could be found, their behaviour in the presence of $C_6H_{13}NMe_3Br$ clearly depended on their age and "history."

Interaction of NTC, ATC, and TYMV RNA with Quaternary Ammonium Salts

In order to get an impression of the cause of the instability of TYMV towards certain quaternary ammonium salts at pH 9, the possible interactions of the intact virus protein and of virus nucleic acid with these salts have been studied. In Fig. 5a is shown the sedimentation pattern of NTC after incubation with $C_8H_{17}NMe_3Br$; Fig. 5b is the same for ATC incubated with $C_6H_{13}NMe_3Br$, and Fig. 5c gives the sedimentation pattern of TYMV, incubated with $C_6H_{13}NMe_3Br$. No appreciable degradation of ATC or NTC, under the circumstances which cause TYMV to decompose rapidly, has been found. In the case of TYMV RNA there has been observed a distinct difference in the sedimentation patterns in the presence of NaBr, $C_4H_9NMe_3Br$ and of $C_6H_{13}NMe_3Br$ (Fig. 6). Although no quantitative results have been obtained, it is clear from the schlieren pictures that the RNA looses its original capacity to sediment as a homogeneous boundary, and more so after interacting with quaternary salts containing the longer straight-chain alkyl groups.

To compare the action exerted on TYMV by quaternary ammonium salts, with other possible ways of virus degradation, we have studied the interaction of several more or less related compounds with the virus. Attention was especially directed to finding

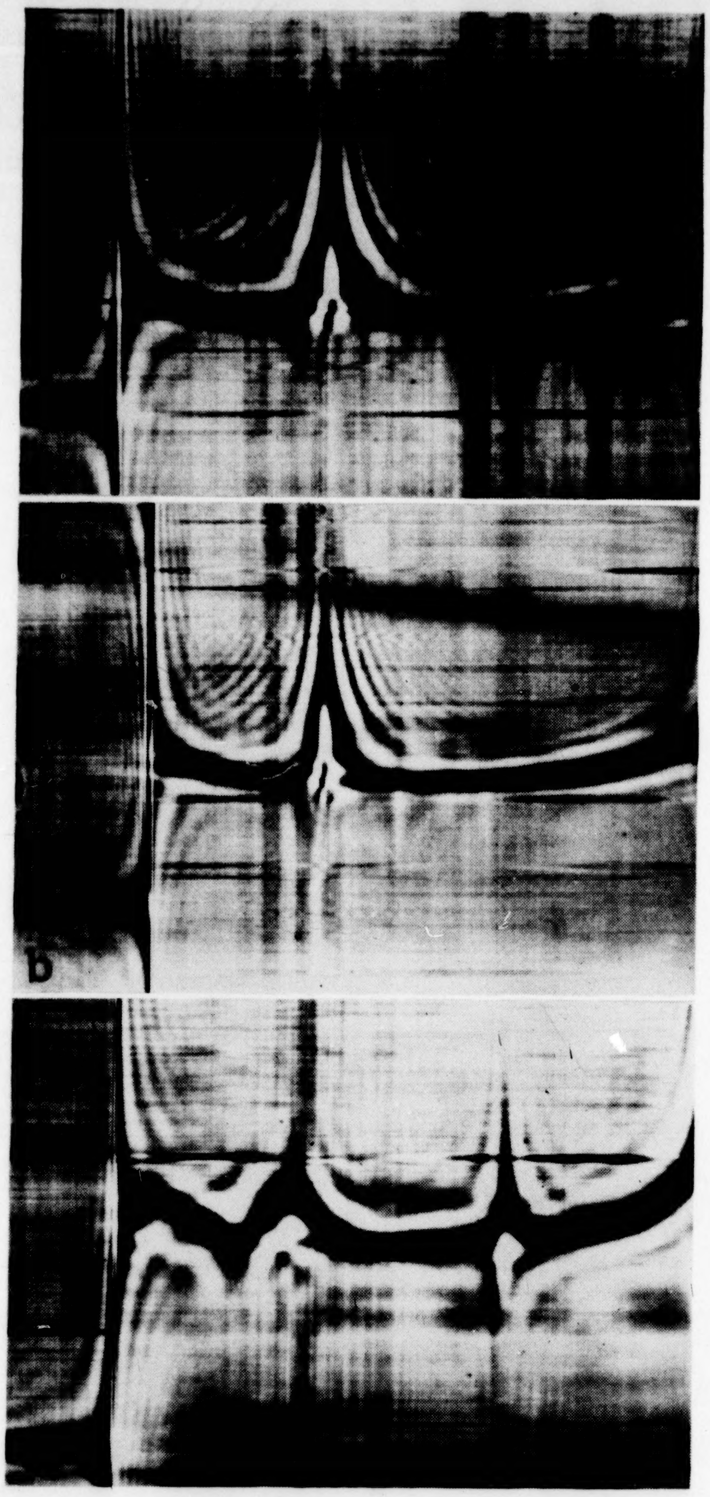

FIG. 5. Interactions of TYMV, NTC, and ATC with quaternary ammonium salts. Sedimentation patterns: (a) 0.54 mg NTC per ml; 0.20 *M* $C_8H_{17}N \cdot Me_3Br$; 105 minutes at pH 9.0 and 20°C. Photograph taken 24 minutes after start of run; speed 29,500 rpm; temp. 20°C; angle of phase plate 40 degrees. (b) 0.72 mg ATC per ml; 0.20 *M* $C_6H_{13}N \cdot Me_3Br$; 120 minutes at pH 9.0 and 20°C. Photograph taken 12 minutes after start of run; speed 42,040 rpm; temp. 20°C, angle of phase plate 50 degrees. (c) 1.4 mg TYMV-VIII per ml; 0.20 *M* $C_6H_{13}NMe_3Br$; 120 minutes at pH 9.0 and 20°C. Photograph taken 12 minutes after start of run; speed 42,040 rpm; temp. 20°C; angle of phase plate 40 degrees. 12-mm standard centrepieces used throughout.

other compounds which would be able to decompose the virus but leave the virus protein shells intact, as do some of the quaternary ammonium salts. Using a negatively charged amphiphilic molecule, like dodecyl sulphate, only traces of decomposition products could be found when the virus was incubated for 2 hours at pH 9. The polyamine spermine was likewise tested: under the same conditions as above only very small amounts of degradation products were formed. *N,N*-Dimethyl-*n*-heptylamine and *n*-heptylamine were incubated with TYMV under the same conditions used for the quaternary ammonium salts. Only the tertiary amine was able to cause degradation of the virus. Since the amine was only slightly soluble at pH 9, no quantitative results could be obtained.

FIG. 6. Interactions of TYMV RNA with quaternary ammonium salts. Sedimentation patterns: (a) 0.55 mg RNA per ml; 0.20 *M* NaBr; 60 minutes at pH 9.0 and 20°C. (b) 0.55 mg RNA per ml; 0.20 *M* $C_4H_9NMe_3Br$; 60 minutes at pH 9.0 and 20°C. (c) 0.55 mg RNA per ml; 0.20 *M* $C_6H_{13}NMe_3Br$; 60 minutes at pH 9.0 and 20°C. Photographs were taken 20 minutes after start of run; speed 50,740 rpm; temp. 20°C; angle of phase plate 50 degrees; 30-mm standard aluminum centrepiece.

DISCUSSION

The degradation of TYMV in the presence of quaternary ammonium salts differs in some respects from the alkaline degradation of the virus as described by Kaper (1964). We did not observe the formation of ATC at pH 9, at least not in large amounts. This could not be improved by adding more salt, be it quaternary ammonium salts or alkali salts. Evidently the presence of the lipophilic alkyl group as well as of the positive charge on the quaternary nitrogen atom is essential in causing the observed effects: amphiphilic compounds with a negative charge like sodium dodecyl sulphate did not cause any appreciable degradation, whereas shortening of the alkyl group in a quaternary ammonium salt from 6 to 5 C-atoms diminished drastically the ability to degrade the virus.

The large influence exerted by the pH on the rate of degradation suggests the importance of the ionic interaction between the reacting substances. It seems obvious that the negative phosphate groups of the nucleic acid and the positive quaternary nitrogen atoms form the interacting groups. A less strongly dissociated salt like a primary or a tertiary amine would not interact so strongly.

The following mechanism is proposed: It seems likely that the quaternary ammonium ions interact with the RNA inside the virus particles forming soluble salt complexes which are less dense and at the same time perhaps heavier than ordinary alkali nucleates. This causes the RNA inside the virus particles to uncoil and expand. At pH 9 the virus protein shell is apparently not flexible enough to let the expanding RNA pass with-

out breaking down. In this way the shell structure is lost, causing degradation and ultimately denaturation of the protein. At pH 12 the shell is indeed capable of expanding reversibly, making possible the escape of the RNA without losing its structure. At this pH the protein will carry predominantly negatively charged groups, which presumably cause the expansion of the shell by means of electrostatic repulsion. Thus ATC can be formed in high yields (Kaper, 1964). The rate of virus degradation is approximately proportional to the concentration of the quaternary ammonium salt, which is present in a hundredfold excess or more (calculated per nucleotide). Furthermore the rate of the reaction is proportional to a power of the virus concentration between 0 and 1, which points to the complicated nature of the reactions taking place. The fact that addition of an alkali salt retards the rate of virus degradation appreciably is an indication that penetration of the positive ions into the virus particle is the rate-determining process. By virtue of their much higher penetration velocity, the concentration of the small sodium ions inside the virus particles increases more rapidly than the concentration of the larger quaternary ammonium ions, thus masking the negative groups which play the most important role in the interactions. There must be two opposite influences exerted by the quaternary ammonium ions which determine the relative rate of degradation: the velocity of penetration and the ability to cause conformational changes in the RNA. It is not likely that in TYMV RNA covalent bonds can be broken by the quaternary ammonium ions (Hirth *et al.*, 1960), thus leaving only conformational changes to cause the polydispersity in the sedimentation coefficient of TYMV RNA which has interacted with quaternary ammonium salts.

The data from Fig. 1 clearly indicate that a precise characterization of the TYMV preparations, used for interactions like those described, is necessary in order to ensure significant and reproducible results.

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RNA-CH_2O reaction product which forms after heating to 96° at 0.02 μ_{Na^+} appears to be structurally stabilized at a T_m of about 33°. This stability may result from the formation of methylene bridges between helical elements of the RNA.

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Transmission of Tobacco Stunt Virus by *Olpidium brassicae*

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When tobacco (*Nicotiana tabacum* L. var. Bright Yellow) was seeded in stunt-infested soil which had dried for 9–12 years, the plants became infected with stunt. Stunt resulted when zoospores or resting sporangia of *Olpidium brassicae* (Wor.) Dang. were transferred from stunt-infected to healthy tobacco, but not when tobacco stunt virus (TSV) alone was added to roots of tobacco seedlings. Stunt did not result if viruliferous zoospores were killed by 10 minutes at 55°C, 24 hours at −10°, 1-phenylthiosemicarbazide, sodium dithiosemicarbazide, oxine, or salicylaldoxime. None of these treatments inactivated virus used as inoculum. A TSV-free strain of *Olpidium* from tobacco became viruliferous when added to roots of tobacco mechanically inoculated with TSV on the leaves. Drainage water from tobacco plants with TSV and *Olpidium* contained *Olpidium* and virus and was infective to healthy tobacco, but the drainage water from plants with TSV without *Olpidium* was not.

INTRODUCTION

The occurrence of tobacco stunt in Japan was first recorded by Nakamura and Tsumagari in 1943. By 1956 the disease had been reported throughout the country and was then seriously damaging tobacco leaf production (Hidaka *et al.*, 1956). The nature of the causal agent was at first in doubt. Hidaka (1951, 1956) reported that the disease was caused by a virus. Inoculation with preparations of viruslike particles was not successful, however, and grafting was the only method of artificial transmission (Hidaka *et al.*, 1956). The virus is soil transmitted; Uozumi (1954) and Hidaka *et al.* (1956) studied various factors affecting the infectivity of stunt-infested soil. Recently Hiruki (1964a) reported that incorporating 8 chelating agents, particularly 1-phenylthiosemicarbazide (1-PTC) in inoculum permitted mechanical transmission of TSV.

In an earlier report (Hidaka *et al.*, 1956) evidence was presented that the disease had a fungus vector. Treating infested soil with O_2 or CO_2 for 40 days had little influence on infectivity. Since tobacco mosaic virus (TMV) in soil is inactivated by aeration (Johnson, 1926; Johnson and Hoggan, 1937), these results were interpreted to mean that TSV remained within the body of a resistant vector rather than being simply adsorbed to soil particles. Furthermore, treatment of infested soil with fungicides reduced infectivity by 90% or more, whereas insecticides had no appreciable effect, suggesting that the virus vector was a fungus. Hidaka (1960) and Hidaka and Tagawa (1962) reported an association of *Olpidium brassicae* (Wor.) Dang. with tobacco stunt and considered the fungus as a possible vector. Hiruki (1964c) recently reported that zoospores of *Olpidium* from TSV-infected tobacco plants acquired tobacco necrosis virus (TNV) *in vitro* and transmitted both viruses to tobacco or mung bean (*Phaseolus aureus* Roxb.).

Discovery of 1-PTC as an agent stabilizing infectivity of TSV and discovery of an assay host, *Chenopodium amaranticolor* Coste & Reyn. (Hiruki, 1964a), made it possible to investigate quantitatively the vector relationship of *Olpidium* in transmission of

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TSV. In this paper evidence of the constant association of *Olpidium* with tobacco stunt and the acquisition of TSV by the fungus are presented.

MATERIALS AND METHODS

In 1959 a strain of *O. brassicae* was isolated from tobacco seedlings (*Nicotiana tabacum* L. var. Bright Yellow) grown in TSV-infested soil collected from a tobacco seedbed of 1954. This isolate is called henceforth the tobacco strain of *Olpidium*. The fungus was maintained in glasshouse-grown tobacco seedlings and used throughout the present investigations. When tobacco seedlings were inoculated with resting sporangia from the air-dried roots, zoosporangia were formed 16–21 days later at 16–17°C. The roots of tobacco infected with the tobacco strain of *Olpidium* associated with TSV ("viruliferous *Olpidium*") were washed quickly and immersed in a small amount of tap water or 0.01 *M* pH 7.0 phosphate buffer in a petri dish. Zoosporangia usually germinated within 5–10 minutes, discharging active zoospores. When the zoospore suspension was poured on pots containing 2- to 3-week-old tobacco seedlings, new zoospores were formed in 3–4 days. After 1–2 weeks, the *Olpidium* had multiplied sufficiently to yield concentrated zoospore suspensions for further experimental purposes. Hemacytometer counts were made at times to estimate zoospore concentration. An isolate of *Olpidium* free from virus was obtained after repeated transfer on cowpea (*Vigna sinensis* (Torner) Savi var. Black Eye), and was maintained in tobacco and cowpea.

Since the symptoms of tobacco stunt are masked at temperatures above 25° (Hidaka and Hiruki, 1958), the experiments were carried out in a glasshouse maintained at 16–21°. Plants were usually grown in sterilized soil in 4-inch porous clay pots which were placed in waterproof glazed pots to avoid contamination of the roots (Yarwood, 1960). For watering, half-strength Hoagland's solution was used.

TSV inoculum was prepared by grinding leaf disks or washed roots in a measured amount of 0.01 *M* pH 6.8 phosphate buffer containing 0.001 *M* 1-PTC (1-PTC phosphate buffer) unless otherwise stated. The 1-PTC phosphate buffer was stored at −10° until use to prevent a rapid color change which occurred on standing at room temperature.

Throughout the present investigation Bright Yellow tobacco was used as an indicator plant for tobacco stunt. *C. amaranticolor* was used for local lesion assay of TSV when plants were 4–5 inches tall and had 4–6 uniform leaves. Lesions were counted 10–14 days after inoculation.

RESULTS

Association of *Olpidium* with TSV

Association in Soil Stored 9–12 Years

Occurrence of tobacco stunt in soil stored 4 years has been reported (Hidaka *et al.*, 1956). The soil samples collected by Uozumi from tobacco seedbeds in Hatano in 1951 and in 1954 were stored in glass beakers with a tight paper seal after air drying. As most of the previously published data were obtained using these samples, it was necessary to determine whether *Olpidium* invariably occurred in the roots of every stunted tobacco plant in order to correlate the presence of the fungus with TSV infection.

Tobacco seedlings in the infested soil or sterilized soil (control) in 8-inch pots were kept at 17°. Examination for *Olpidium* infection was made at various time intervals and it was first recognized 21 days after the start of the experiment. Active and numerous zoospores were liberated from shiny, round or rod-shaped zoosporangia in the washed roots within 5 minutes after immersion in phosphate buffer. The number of zoospores increased with time of soaking up to 30 minutes. Vein clearing of the seedlings first occurred 26 days after seeding. When definite stunt symptoms developed each seedling was carefully removed to examine the roots for *Olpidium* infection and to assay TSV infectivity of the leaves. Five leaf disks, 8 mm in diameter, from each plant were ground in 0.5 ml of 1-PTC phosphate buffer and inoculated to a *C. amaranticolor* plant. Table 1 summarizes the data on stunt symptom expression, virus recovery from the leaf, and root infection with *Olpidium*. A close associa-

TABLE 1

NUMBER OF PLANTS INFECTED WITH *Olpidium* AND TOBACCO STUNT IN AIR-DRIED SOIL STORED 9–12 YEARS[a]

Infested soil		Number of plants			
Years stored	Steam sterilized	Inoculated	Stunted	TSV-infected[b]	*Olpidium*-infected
9	No	35	26	28	35
12	No	35	25	26	29
—	Yes	35	0	0	0

[a] Date of seeding, March 4, 1963; date of final recording of tobacco stunt and *Olpidium* infection, April 9, 1963.

[b] Assays were made on *C. amaranticolor*.

tion is indicated between *Olpidium* and TSV infection.

In another experiment, the viruliferous *Olpidium* had been maintained since 1959 in tobacco seedlings by occasional transfers by means of zoospore suspensions of the fungus, that is, for 5 years. During the serial transferring of the viruliferous *Olpidium*, the fungus was constantly associated with tobacco stunt. Without exception, the plants developing symptoms of tobacco stunt showed *Olpidium* in the roots. TSV infectivity was also detected in extracts from leaves and roots by mechanical inoculation to *C. amaranticolor*.

Infectivity of Heated Olpidium

A preliminary experiment indicated that the thermal inactivation point of TSV was 75–80° for 10 minutes (Hiruki, unpublished data). On the other hand, *Olpidium* was inactivated by heating to 55° for 10 minutes (Grogan *et al.*, 1958; Campbell and Grogan, 1964), and lost its ability to transmit TNV to mung bean by heating to 50° for 10 seconds (Teakle, 1962). Heating viruliferous zoospores at 45° for 20 minutes and viruliferous resting spores at 55° for 20 minutes prevented occurrence of tobacco stunt and prevented infection of *Olpidium* in the roots (Hidaka and Tagawa, 1962). In the present work, an attempt was made to correlate infection by the heat-treated *Olpidium* with TSV infection in the roots and with the occurrence of tobacco stunt.

Portions of 5 ml of a viruliferous zoospore suspension (8.2×10^5 zoospores per milliliter) in small glass tubes (5×50 mm) were heated at 35°, 40°, 45°, 50°, 55°, 60°, and 65° for 10 minutes. Immediately after heating, the suspensions were cooled in an ice bath and then poured onto potted soil in which 20 tobacco seedlings had been transplanted. Thereafter, the roots and root-washings were examined at various time intervals for *Olpidium*. At 1–3 weeks after the start of the experiment, a number of zoospores were observed in the root washings. At this time, early symptoms of stunt seemed to be developing on some plants.

TABLE 2

EFFECT OF HEAT TREATMENT OF *Olpidium* ZOOSPORES ON THE OCCURRENCE OF TOBACCO STUNT AND TSV RECOVERY

Temperature (°C)	Motility of zoospores after treatment	Occurrence of tobacco stunt[a]	Infection by *Olpidium*	Recovery of TSV infectivity[b]
35	±	13/20	++	26
40	−	10/20	+	23
45	−	10/20	+	15
50	−	2/20	+	10
55	−	0/20	−	0
60	−	0/20	−	0
65	−	0/20	−	0
Control[c]	++++	18/20	++++	28

[a] Two-week-old tobacco seedlings were inoculated. Numerators are number of plants infected and denominators are number of plants tested.

[b] Total number of local lesions on 4 leaves of *C. amaranticolor*.

[c] Room temperature at the time of the experiment was 21°C.

Final recording of stunt and infectivity tests were made 50 days after inoculation with the zoospores.

The results (Table 2) clearly indicate that the tobacco strain of *Olpidium* survived heating to 50° for 10 minutes and transmitted TSV, but did not survive at 55° or above. It is also shown that TSV infectivity of root extracts and occurrence of stunt coincided with *Olpidium* infection.

Infectivity of Frozen Olpidium

Freezing kills *Olpidium* zoospores but not resting sporangia, whereas TSV remains infective at −25° for at least 40 days (Hiruki, unpublished data). Therefore, it appeared possible to eliminate the motile zoospores from the TSV–*Olpidium* complex by freezing, and to determine the role of *Olpidium* as a vector in transmitting TSV.

The resting sporangia suspension was prepared from 1 g of roots stored for 2 months after being heavily infected for 3 months with viruliferous *Olpidium*. These roots were immersed and ground in 10 ml of phosphate buffer in a mortar. Of the suspension of active zoospores, 10 ml (5.6×10^5 zoospores per milliliter) was filtered through Whatman no. 1 filter paper. One half of the suspension was frozen at −10° overnight and the other half served as control by inoculating a lot of 10 tobacco seedlings immediately after the suspensions had been prepared. Recording of symptoms, examination for *Olpidium* infection, and assays of TSV infectivity were made 45 days after inoculation.

The frozen zoospores were disintegrated and did not transmit TSV, whereas among controls 9 out of 10 plants were infected by motile and viruliferous zoospores, 7 out of 10 by frozen and viruliferous resting sporangia, and 8 out of 10 by unfrozen and viruliferous resting sporangia. TSV infectivity was consistently detected by inoculating *C. amaranticolor* with the leaf and root extracts of stunted plants. These results indicate that *Olpidium* infection is correlated with occurrence of stunt and TSV recovery from the infected plants.

Infectivity of Centrifuged Olpidium

A preliminary experiment indicated that *Olpidium* zoospores and resting sporangia in suspensions of root extracts were removed by low speed centrifugation, and TSV infectivity could be detected in the supernatant of such extracts. In this experiment, therefore, transmission of TSV with or without the presence of *Olpidium* zoospores or resting sporangia was investigated by means of centrifugation.

Tobacco stunt-affected plants approximately 2-month-old, raised in infested soil, served as inocula. There were 9 treatment groups: (1) A 10-g sample of the roots was immersed in 100 ml of phosphate buffer for 20 minutes; this was then filtered through a double layer of gauze. One half of the filtrate, containing TSV and zoospores, was saved. (2) The other half was centrifuged at 4000 rpm for 30 minutes; 25 ml of the supernatant was carefully taken by a syringe from the top of the centrifuge tube (Lusteroid tube for Spinco model L rotor no. 21) and saved. (3) The remaining bottom 25 ml and the pellet, containing TSV and zoospores, were saved. (4) Another 10 g of the roots frozen at −25° overnight was ground with a mortar and pestle. The slurry was diluted in 100 ml of phosphate buffer and filtered through a double layer of gauze. One half of the filtrate containing TSV and resting sporangia was saved. The other half was centrifuged in similar manner as (2) and divided into (5) the top 25 ml of the supernatant, and (6) the bottom 25 ml including the pellet containing TSV and resting sporangia. As controls, (7) leaf extract in 1-PTC phosphate buffer, (8) leaf extract in phosphate buffer, and (9) phosphate buffer alone were tested.

Of each 25 ml suspension listed above, 5 ml was saved for inoculating *C. amaranticolor* mechanically. The remaining 20 ml was poured onto soil around lots of 30 tobacco seedlings. To determine *Olpidium* infection, 10 seedlings were taken from each treatment 17 days after inoculation. Twenty other seedlings were saved for a further 2 weeks to observe the occurrence of stunt and determine TSV infection. The results of two experiments summarized in Table 3 show that tobacco stunt was induced only when zoospores or resting sporangia of *Olpidium* were transferred from stunt-infected to

TABLE 3

REMOVAL OF ZOOSPORES AND RESTING SPORANGIA OF *Olpidium* FROM ROOT EXTRACTS BY CENTRIFUGATION AND ITS EFFECT ON OCCURRENCE OF TOBACCO STUNT

Inoculum from tobacco stunt plants[a]	Treatment	TSV infectivity of inoculum[b]		No. of plants infected with *Olpidium*[c]		No. of plants infected with tobacco stunt[c]	
		Expt.		Expt.		Expt.	
		I	II	I	II	I	II
(1) Zoospores	Not centrifuged	3	4	5/10	5/10	10/20	16/20
(3) Zoospores	Centrifuged pellet	8	14	7/10	6/10	20/20	18/20
(4) Resting sporangia	Not centrifuged	0	—	3/10	—	20/20	—
(6) Resting sporangia	Centrifuged pellet	3	—	5/10	—	20/20	—
(2) Zoospores	Zoospores removed by centrifugation	4	18	0/10	0/10	0/20	0/20
(5) Resting sporangia	Resting sporangia removed by centrifugation	6	—	0/10	—	1/20[d]	—
(7) Leaf extract	1-PTC	268	230	0/10	0/10	0/20	0/20
(8) Leaf extract	Phosphate buffer	92	2	0/10	0/10	0/20	0/20
(9) Control	Phosphate buffer	0	0	0/10	0/10	0/20	0/20

[a] See text. Numbers in parentheses refer to treatment groups.

[b] Total number of local lesions on 10 (experiment I) or on 24 (experiment II) half-leaves of *C. amaranticolor*, respectively.

[c] Two-week-old tobacco seedlings were inoculated. Numerators are number of plants infected and denominators are number of plants tested.

[d] Possibly due to contamination.

healthy tobacco, but not when TSV alone was transferred.

Infectivity of Chemically Treated Olpidium

Chelating agents such as 1-PTC, sodium diethyldithiocarbamate (NaDIECA), salicylaldoxime, nitrophenol, sodium cyanide, cupferron, oxine, and thioglycolic acid stabilize TSV infectivity (Hiruki, 1964a). The fungicidal action of some of these compounds has also been reported (Zentmyer, 1943; Gausman *et al.*, 1953; Ludwig and Thorn, 1960). Therefore, it was of interest to investigate the effect of six of these chemicals on TSV transmission by zoospores.

One volume of a solution of each chemical was added to 9 volumes of a viruliferous zoospore suspension (2.5×10^5 zoospores per milliliter) to give a final concentration of 0.001 *M*. This was allowed to stand for 30 minutes. Twelve tobacco seedlings were added to each solution, allowed to stand a further 30 minutes, and then transplanted to sterile soil. The 1-PTC and cupferron immobilized the zoospores immediately. NaDIECA, salicylaldoxime, and oxine moderately suppressed zoospore motility, and finally immobilized them by the end of the treatment. The 1-phenyl-2-thiourea (1-PTU) showed only slight effect on motility. NaDIECA, 1-PTC, salicylaldoxime, oxine, and cupferron eliminated *Olpidium* infection and transmission of TSV. The 1-PTU reduced TSV transmission greatly. Multiplication of 1-PTU-treated *Olpidium* was also poorer than in controls. These results (Table 4) show that the *Olpidium* infection is closely correlated to transmission of TSV.

ACQUISITION AND TRANSMISSION OF TSV BY *Olpidium*

Investigating acquisition of TSV by *Olpidium* became possible when the fungus free from virus was isolated by repeated inoculations of the tobacco strain of *Olpidium* to Black Eye cowpea seedlings. In subsequent

TABLE 4

EFFECT OF CHEMICAL TREATMENT OF *Olpidium* ZOOSPORES ON OCCURRENCE OF TOBACCO STUNT AND TSV RECOVERY

Chemical added to viruliferous zoospore suspension	Motility of zoospores after treatment[a]	Occurrence of tobacco stunt[b]	Infection by *Olpidium*	Recovery of TSV infectivity[c]
1-PTC	−	0/12	−	0
Cupferron	−	0/12	−	0
NaDIECA	+	0/12	−	0
Salicylaldoxime	+	0/12	−	0
Oxine	++	0/12	−	0
1-PTU	+++	3/12	+	11
None	++++	11/12	++++	62

[a] Recorded 15 minutes after the treatment.

[b] Twenty-day-old tobacco seedlings were inoculated. Stunted plants were recorded 47 days after inoculation. Numerators are number of plants infected and denominators are number of plants tested.

[c] Total number of local lesions on 4 leaves of *C. amaranticolor*. Root extracts from each lot of 12 plants served as inocula.

serial transfers to healthy tobacco seedlings the isolate consistently failed to induce stunt, but infected the roots of tobacco and multiplied abundantly. TSV infectivity was also checked by inoculating *C. amaranticolor* with root extracts, which were found free from the virus. The isolate, "virus-free *Olpidium*," was used in the following experiments to determine its ability to transmit TSV.

Olpidium Inoculation of TSV-Infected Plants

Five leaf tobacco plants, infected 11 days with TSV, were inoculated with 10 ml (9.4×10^4 zoospores per milliliter) of the virus-free *Olpidium* zoospore suspension when they were showing chlorotic spots or rings on the inoculated leaves and systemic veinclearing. The following treatment groups were included as controls: (1) healthy plants inoculated with the virus-free *Olpidium*, (2) uninoculated healthy plants, (3) plants mechanically inoculated with TSV and showing typical symptoms, (4) plants inoculated with the viruliferous *Olpidium*. Ten days after inoculation with *Olpidium*, the roots from 5 different treatment groups were immersed in 5 ml of phosphate buffer for 15 minutes to obtain zoospores. Twenty 2-week-old tobacco seedlings were then inoculated by dipping for 1 hour. The seedlings were transplanted and examined for stunt 20 days after inoculation. Stunt developed only from inoculations with a combination of TSV and *Olpidium*. TSV was transmitted to *C. amaranticolor* only from such plants.

TSV Inoculation of *Olpidium*-Infected Plants

Similar experiments were carried out using tobacco seedlings inoculated with the virus-free *Olpidium*. Two leaves of each plant were inoculated with TSV 6 days after inoculation of the roots with the *Olpidium* zoospores. Three weeks later when systemic symptoms were clearly developed, the roots were washed and immersed in phosphate buffer to prepare a zoospore suspension. The controls included were similar to those of the previous experiment. Stunt again developed only from inoculum taken from plants with TSV and *Olpidium*. TSV was also recovered only from the plants inoculated with TSV and *Olpidium*.

These results conclusively indicate that the virus-free *Olpidium* acquired TSV when the host plant, whose roots were infected with the *Olpidium*, was inoculated with TSV.

Drainage Water from TSV-Infected Plant

Natural release of TMV and TNV from the living roots has been reported (Yarwood, 1960), suggesting that drainage water from infected plants may play an important role in natural dissemination of virus.

It was of interest to determine whether TSV infectivity could be detected in the drainage water from recently inoculated plants and to determine whether this is re-

TABLE 5

TRANSMISSION OF TSV BY *Olpidium* IN DRAINAGE WATER COLLECTED FROM TOBACCO STUNT PLANTS

Tested plants infected with	Infectivity of[a] The extract of the root which provided drainage water	Infectivity of[a] Drainage water	*Olpidium* in drainage water	Occurrence of tobacco stunt[b]	Recovery of TSV infectivity[c]
TSV	23	0	−	0/20	0
TSV and nonviruliferous *Olpidium*	13	0	+	18/20	38
Nonviruliferous *Olpidium*	0	0	+	0/20	0
Uninoculated	0	0	−	0/20	0

[a] Total number of local lesions on 20 half-leaves of *C. amaranticolor*.

[b] Twelve-day-old tobacco seedlings were inoculated. Numerators are number of plants infected and denominators are number of plants tested.

[c] Total number of local lesions on 8 half-leaves of *C. amaranticolor*. Root extracts from each lot of 20 plants served as inocula.

lated to transmission of TSV by *Olpidium*. The treatment groups involved were: (1) plants inoculated with TSV only, (2) plants inoculated with TSV and then 11 days later inoculated with the virus-free *Olpidium*, (3) plants inoculated with the virus-free *Olpidium* only, and (4) uninoculated plants. Twenty-one days after inoculation with TSV (10 days after *Olpidium* inoculation), drainage water from the plants receiving each treatment was collected into a small vial. This was done by applying 100 ml of phosphate buffer from a siphon to the base of the stem, drop by drop, during 30 minutes. The pots had been carefully kept fairly dry before they received the buffer. The first 3 ml of the drainage water coming from the bottom hole of the pot were saved for assay. Inoculations with the drainage water on *C. amaranticolor* gave entirely negative results. In the drainage water from treatment groups (2) and (3) motile zoospores were observed with a microscope. After the drainage water had been collected, the roots were washed and ground in 1-PTC phosphate buffer. TSV infectivity was found in roots from treatment groups (1) and (2). However, transmission of TSV was obtained only from plants that had been infected with TSV and *Olpidium* (treatment 2). Transmission was obtained by inoculating 20 tobacco seedlings by dipping them in the drainage water for 1 hour and then transplanting them to soil, and the transmission was checked by infectivity tests on *C. amaranticolor* using root extracts from the tobacco seedlings (Table 5).

In another experiment, the drainage water was collected from a plant infected with TSV for 20 days in order to make a series of infectivity tests. Successive collections of six inocula were made from continuous flow of the droplets. Each inoculum, in which no *Olpidium* zoospores were detected microscopically, consisted of 10 drops obtained in approximately 1 minute. Assays on *C. amaranticolor* for TSV were entirely negative.

In another experiment, the drainage water, which was free from *Olpidium*, was collected from the plants infected with TSV for 25 days. The zoospores were obtained by immersing the roots of the plants infected with virus-free *Olpidium* in this drainage water. The test seedlings were then dipped into the preparation for 1 hour and transplanted. However, no transmission of TSV occurred.

DISCUSSION

Experimental results have indicated a close association of *Olpidium* with tobacco stunt. A tobacco strain of *Olpidium* and TSV were recovered in association from soil stored 9–12 years in an air-dry condition in which the *Olpidium* remained as resting sporangia through the period of the storage (Table 1). In case of lettuce big-vein it has been reported that the causal agent was carried in soil as long as 8 years (Pryor, 1946). TNV, which is another virus trans-

missible by *Olpidium*, was also isolated in association with resting sporangia obtained from air-dried soil which was collected from a tobacco field and stored for 4 months (Hiruki, unpublished data). Therefore, it can be concluded that the *Olpidium* carries TSV in a very stable state, whatever the mechanism may be. Furthermore, this stability seems to be one of the most specific features of virus transmission by *Olpidium*. The close association between TSV and the *Olpidium* has been maintained in tobacco for 5 years without any loss of TSV infectivity by serial inoculation with viruliferous zoospores. On this particular point, there is some similarity between TSV and lettuce big-vein virus (LBVV). In the case of TNV, the virus could be maintained in lettuce roots in association with *Olpidium* (Teakle, 1963; Fry and Grogan, 1964; Hiruki, unpublished data), but TNV titer fell to low levels in successive transfer of *Olpidium* zoospores (Fry and Grogan, 1964).

Tobacco stunt was consistently induced as a result of *Olpidium* infection, and TSV was detected in the leaves and roots of the stunted plants. However, virus transmission was prevented by physical treatments which did not affect TSV infectivity but killed or removed zoospores of *Olpidium*, for example, heating, freezing, or centrifugation; or by chemical treatments which stabilized TSV infectivity (Hiruki, 1964a), but killed the zoospores (Table 4) (Hiruki, 1964b). Even highly infectious preparations of TSV induced no stunt when added to roots without *Olipidium*. These data strongly indicate that *Olpidium* is the agent responsible for TSV transmission and can be properly called the vector of TSV.

A tobacco strain of *Olpidium* carrying TSV could be freed from the virus by serial transfer of the zoospores on cowpea. The same phenomenon was reported in the transmission of LBVV during continued culture on sugar beet (Campbell, 1962) and *Plantago major* or *Veronica persica* (Tomlinson and Garrett, 1964). These results suggest some intimate interrelationships between virus, *Olpidium* as a vector, and their host plant, although the mechanism by which the fungus is freed from virus is not clear. Evidence has also been presented which shows that the zoospores of virus-free *Olpidium* can acquire TSV and transmit it to the roots of tobacco seedlings which they infect. The virus-free *Olpidium* which had been multiplying in the roots became viruliferous after the leaves were mechanically inoculated with TSV. The *Olpidium* also acquired TSV and became viruliferous when the zoospores were introduced to the roots of TSV-infected plants. However, when the virus-free *Olpidium* zoospores were introduced into the drainage water from TSV-infected roots and test seedlings were inoculated by dipping them into the preparation, no TSV transmission occurred. This result indicates that the intimate contact of zoospores with a sufficient amount of TSV is necessary for successful transmission. Apparently any free TSV that might be released into the root drainage water seems to be unimportant in TSV transmission. The mechanism by which *Olpidium* acquires TSV *in vivo* is unknown. Further information on virus acquisition by *Olpidium* will become available when purification of TSV, now in progress, is accomplished. It might be supposed, according to these results, that *Olpidium* protoplasm forms an intimate combination with TSV shortly after virus contact with it and that the direct contact of *Olpidium* protoplasm to the host cytoplasm makes it possible to successfully transfer TSV from the fungus to the host or vice versa. This might occur at a very early stage of *Olpidium* infection before a cell wall has formed outside the fungus protoplast, as suggested in case of LBVV transmission (Campbell and Grogan, 1964). Thus, it might be possible for the fungus to carry virus in a very stable state.

The results of the present investigation established that TSV and the *Olpidium* form a specific and intimate relationship when they come into contact; the zoospores can acquire and transmit TSV as an efficient vector, and when the resting sporangia are formed the infectivity of TSV can be stably preserved in close association with the fungus. In this context, the association of the *Olpidium* with TSV seems to be one of the most stable, and efficient means of virus

acquisition, preservation, and transmission in soil.

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The Use of Bentonite in the Purification of Plant Viruses

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Tests were made on the affinity of the major normal particulate components of sap and of a number of plant viruses for magnesium bentonite in solutions containing various concentrations of $MgSO_4$ and Na-K phosphate buffer, pH 7.4. Ribosomes, 18 S protein, and the green material in sap were all readily absorbed by bentonite in solutions containing magnesium at a concentration of $10^{-3}M$ or more. Several viruses, however, were not adsorbed under these conditions, so methods for purifying a number of them were developed using bentonite in combination with differential centrifugation. Viruses such as those of turnip yellow mosaic and carnation mottle were readily purified using bentonite in $5 \times 10^{-3}M$ magnesium, but others such as tobacco mosaic virus and wild cucumber mosaic virus were adsorbed under these conditions, and a magnesium concentration of $10^{-3}M$ had to be used. In the purification of the bean form of tobacco mosaic virus it was necessary to use an even lower concentration of magnesium, but under these conditions the usefulness of the bentonite was reduced. All strains of one virus did not necessarily have the same affinity for bentonite and there was also an adsorption differential between the top and bottom components of wild cucumber mosaic virus. The main advantages of using bentonite in the purification of viruses are that time is saved in the operation and contamination of the product with ribonuclease is reduced or eliminated.

INTRODUCTION

The use of magnesium-treated bentonite in the preparation of ribosomes from rat tumours has been described by Petermann and Pavlovec (1963). While trying to apply this method to the preparation of ribosomes from plant leaves, we observed that they were readily adsorbed by magnesium bentonite in magnesium concentrations greater than 5×10^{-4} *M*. Preliminary results suggested that some plant viruses were not adsorbed under similar conditions. As bentonite has been used to prepare RNA which is free from ribonuclease (Brownhill *et al.*, 1959; Fraenkel-Conrat, *et al.*, 1961; Huppert and Pelmont, 1962), we examined the possibility that the magnesium bentonite could be used to free virus from both ribosomes and from ribonuclease, which has been found to be present in virus preparations (Pirie, 1956). Further work showed that not only were these components removed from sap, but the large amount of protein sedimenting at 18 S (Singer *et al.*, 1952), and the small chlorophyll-containing particles were also adsorbed by the bentonite. We have therefore measured the affinity of a number of viruses for bentonite under a variety of conditions and as a result have been able to develop methods that allow some of them to be prepared rapidly and with little contamination by other sap components.

MATERIALS AND METHODS

Preparation of Bentonite

Magnesium bentonite was prepared from a technical grade bentonite powder (British Drug Houses) by a modification of the method of Petermann and Pavlovec (1963). A considerably higher yield of material was obtained by reducing the speed and duration of the low speed centrifugation step so that only the large dark brown particles

were removed. Initially preparations were made by treating the bentonite with 10^{-3} *M* $MgSO_4$–10^{-3} *M* phosphate buffer,[1] pH 7.4. The analytical data of Petermann and Pavlovec (1963) suggested that about 200 ml of this buffer would be required to saturate 1 g bentonite with magnesium. In agreement with this we observed that a preparation made using only 40 ml of the buffered magnesium solution per 1 g bentonite was much less effective in adsorbing ribosomes than one made using the larger volume of buffer. To reduce the volume of the suspending medium we tried increasing the magnesium and phosphate concentrations to 10^{-2} *M* for the initial suspension and found that the product had a similar adsorbing capacity for ribosomes to that prepared entirely in the more dilute buffer. The following procedure was therefore adopted. Bentonite (100 g) was suspended in 2 l of 10^{-2} *M* $MgSO_4$–10^{-2} *M* phosphate buffer. The mixture was shaken for 2 hours. It was then centrifuged for 1 minute at 1500 rpm (400 *g*) in a MSE Major Centrifuge in 50-ml centrifuge tubes. The resulting supernatant solution was centrifuged for 15 minutes at 12,000 rpm (17,000 *g*) in 50-ml tubes in the SS 34 rotor of a Servall RC2 refrigerated centrifuge, and the supernatant was discarded. The pellets were added to 1 l of 10^{-3} *M* $MgSO_4$–10^{-3} *M* phosphate buffer and shaken again for 2 hours. The low and high speed centrifugations were then repeated and the pellets finally suspended in about 500 ml of the 10^{-3} *M* salt mixture. Dry weight determinations were made on the bentonite in this suspension. For this it was found convenient to precipitate the bentonite from samples by the addition of an equal volume of 90% ethanol. It could then be pelleted by centrifuging at 2500 rpm for 10 minutes. The pellets were washed once with 90% ethanol, dried overnight at 105°, and weighed. Yields varied from 27–35% of the starting material. The bentonite suspensions were adjusted so as to contain 50 mg bentonite per milliliter and stored at 5° until required. Unless otherwise stated, all references to bentonite in this communication are to the magnesium form prepared in this way, and all quantities are given as volumes of preparation A (see below) at 50 mg/ml or as the dry weight of bentonite in the final mixture.

In order to determine whether different bentonite preparations had similar adsorbing capacities, they were tested using Chinese cabbage ribosomes and the procedure outlined later. The test solutions contained per milliliter 1 mg bentonite and 0.2–0.9 mg ribosomes in 10^{-3} *M* $MgSO_4$–2×10^{-3} *M* phosphate buffer. Under these conditions 30–75% of the ribosomes were adsorbed and, as shown in Fig. 1, we found that the adsorbing capacity of each bentonite preparation varied approximately linearly with the ribosome concentration over the range tested. Figure 1 also shows that the three bentonite preparations differed from each other in their adsorbing capacity. This could be due to the preparations containing different proportions of the very fine particles of bentonite which we have shown have a higher adsorbing capacity than the coarser material. In these tests different preparations of ribosomes gave very similar results; this was true whether they had been prepared as described

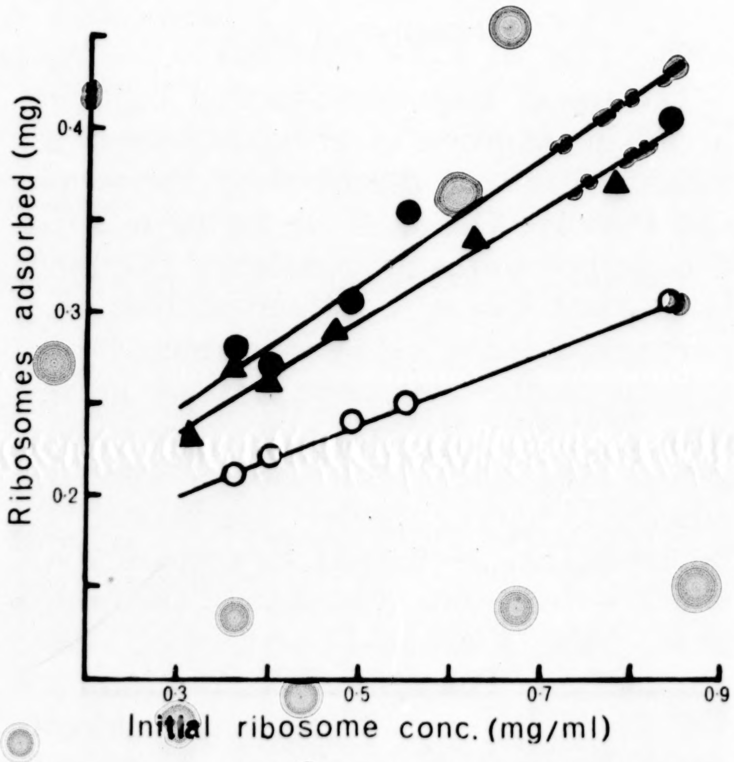

FIG. 1. The effect of concentration of ribosomes on adsorption by magnesium bentonite. The bentonite preparations (A, ●; B, ▲; C, ○) were used at a concentration of 1 mg/ml in the presence of $10^{-3}M$ $MgSO_4$–$2 \times 10^{-3}M$ phosphate.

[1] All phosphate buffers used in this work consisted of a mixture of Na_2HPO_4 and KH_2PO_4.

in the following section or further purified using bentonite (see Results). Preparation A, which had an adsorbing capacity of 0.32 mg ribosomes per milligram of bentonite at a ribosome concentration of 0.5 mg/ml, was the one mainly used in the present work, and all quantities of bentonite given below relate to this preparation. Where other preparations were employed, the quantities used are not stated; instead the quantity of preparation A having the same ribosome-adsorbing capacity is given.

Treatment of the magnesium bentonite with Na_2EDTA (disodium salt of ethylene diaminetetraacetic acid adjusted to pH, 7.6 with NaOH) removes most of the magnesium (Petermann and Pavlovec, 1963), which is replaced by sodium. This method was used to prepare sodium bentonite for comparison with the magnesium form. Magnesium bentonite was centrifuged out of the magnesium-containing solution and resuspended in excess 10^{-2} *M* Na_2EDTA-10^{-2} *M* phosphate buffer, pH 7.4. The bentonite was then centrifuged off and the Na_2EDTA treatment repeated. After another centrifugation, the bentonite pellets were resuspended in the original volume of water. For comparison equal volumes of this material and the magnesium bentonite were used.

Viruses and Hosts

Table 1 shows the virus host combinations used in the present work, which was carried out during the summer and autumn months. The host plants were grown in greenhouses or frames, in pots containing John Innes potting compost. Most of the viruses were from stocks maintained at the laboratory or were currently being studied. The necrotic strain of the turnip yellow mosaic virus had been isolated by one of us (J. H. H.) from the Rademacher strain of the virus sent from Germany. The tobacco necrosis virus was obtained from tobacco, and its relationship to other viruses in this group was unknown. The potato virus X was kindly supplied, in leaves of potato, by Mr. D. E. Richardson of the National Institute of Agricultural Botany, Cambridge. Among the viruses currently being worked on in the laboratory, the following serological relations have been recently determined:

TABLE 1

HOSTS AND VIRUSES USED

Virus	Host
Bean pod mottle virus	French bean (*Phaseolus vulgaris* L. var Tendergreen)
Broad bean mottle virus	Broad bean (*Vicia faba* L.)
Carnation mottle virus	Sweet William (*Dianthus barbatus* L.)
Cocoa yellow mosaic virus	*Chenopodium amaranticolor* Coste & Reyn.
Cowpea mosaic virus (Surinam strain)	Cowpea (*Vigna sinensis* Savi var. Blackeye).
Potato virus X	Potato (*Solanum tuberosum* L.)
Sowbane mosaic virus	*Chenopodium murale* L.
Tobacco mosaic virus	
a. Type strain	Tobacco (*Nicotiana tabacum* L. var. White Burley)
b. Berkeley type strain	Tobacco
c. Cowpea strain (bean form)	French bean (*Phaseolus vulgaris* L. var. Prince)
d. Tomato mosaic virus	Tomato (*Lycopersicon esculentum* Mill. var. Kondine Red)
Tobacco necrosis virus	Tobacco
Tobacco rattle virus (Belladonna strain)	Tobacco
Tomato bushy stunt virus	Thorn apple (*Datura stramonium* L.)
Turnip crinkle virus	Chinese cabbage (*Brassica chinensis* L.)
Turnip yellow mosaic virus	
a. Cauliflower strain	Chinese cabbage
b. Necrotic strain	Chinese cabbage
Wild cucumber mosaic virus	Pumpkin (*Cucurbita pepo* L.)

the Surinam strain of cowpea mosaic virus is related to bean pod mottle and broad bean mottle viruses (van Velsen and Markham, personal communication), the sowbane mosaic virus to carnation mottle virus, and the cocoa yellow mosaic virus to wild cucumber mosaic virus (MacLeod, 1963). Confirmation of the identity of each of the viruses used was found in the value for the sedimentation constant, which was in all cases near the known value.

Infectivity Assay

The infectivity of preparations made with and without the use of bentonite were compared by inoculating onto suitable local-lesion hosts. Tobacco mosaic virus preparations were compared on *Nicotiana glutinosa* L., turnip yellow mosaic virus on Chinese cabbage, and carnation mottle virus on *Chenopodium amaranticolor* Coste & Reyn. Comparisons were made by inoculating a half of every test leaf with the control preparation at a suitable dilution (one that gave 10–50 lesions per half-leaf). A twofold dilution series of the bentonite-prepared virus was then inoculated on the opposite half-leaves so that this preparation at each dilution was compared with the control preparation on about 16 leaves. Equal numbers of right-hand half-leaves and left-hand half-leaves were rubbed with each inoculum to reduce bias due to the inoculation technique. The dilution series was made so that the middle dilution contained virus at the same concentration as that in the control inoculum.

Preparative Methods

Ribosomes, 18 S protein, and viruses were all prepared with the aid of differential centrifugation. Petioles and midribs were removed from leaves before the tissue was ground in a mortar with a little acid-washed sand and the sap was expressed through muslin. The sap was then subjected to one or more cycles of low and high speed centrifugation. Except where stated to have been otherwise, low speed centrifugation was carried out in a Servall RC2 centrifuge using 15-ml tubes in the SS 34 rotor run at 15,000 rpm for 15 minutes. High speed centrifugation was carried out in a Spinco model L centrifuge either in a no. 50 rotor run at 45,000 rpm normally for 1 hour or in a no. 40 rotor at 36,000 rpm for 1.5 hours. All materials and containers were chilled in ice before use and centrifuges were run at about 4°. Modifications of this general method of preparation were adopted where necessary or convenient. Examination of all preparations was made in a Spinco model E analytical ultracentrifuge using either the schlieren optics or the ultraviolet optics as modified by Markham (1963).

18 S protein. This protein was prepared from the leaves of Chinese cabbage plants. The pellets after the first high speed centrifugation at 45,000 rpm for 2 hours were suspended in a volume of $10^{-4}\,M$ $MgSO_4$–$2 \times 10^{-4}\,M$ phosphate equal to that of the sap, and 0.04 ml of bentonite preparation was added per milliliter of suspension. The bentonite was then removed by a low speed centrifugation. This treatment removed from the preparation the green particles and some of the 18 S protein. The resulting preparation contained some ribosomes, but as there was very much more 18 S protein, they were ignored in tests made using this material.

Ribosomes. Most of the tests were made on ribosomes from Chinese cabbage leaves, but tests were also made on ribosomes from the leaves of bean and tobacco. All were made using two cycles of low and high speed centrifugation. After both the high speed centrifugation steps the pellets were suspended in $10^{-4}\,M$ $MgSO_4$–$2 \times 10^{-4}\,M$ phosphate which had a volume equal to one-half to one-fourth that of the original sap, and the final suspension was treated with bentonite as in the preparation of 18 S protein. This resulted in a preparation of ribosomes free from 18 S protein. Most of the particles in such a preparation sedimented at 80 S, but a small proportion sedimenting at 125 S was always present. The 65 S particles sometimes present in sap were never seen in the final preparation.

Viruses. The virus preparations used in tests on their affinity for bentonite needed to be free of appreciable contamination by 18 S protein and ribosomes. This was

necessary for two reasons: firstly these components might adsorb preferentially onto the bentonite and reduce or prevent adsorption of the virus; secondly, if ribosomes were present they might be confused with virus in the analytical ultracentrifuge and they would be certain to interfere where estimation of the virus was based on measurement of the optical density. Most of the viruses were prepared using two cycles of differential centrifugation, during which most of the 18 S protein was lost.

As will be shown later, many of the viruses we used had a lower affinity for bentonite than had the 18 S protein and could be freed of the latter by using bentonite in the presence of magnesium and phosphate at suitable concentrations. After high speed centrifugation the pellets were suspended in the appropriate salt solution, 0.04–0.1 ml of the bentonite preparation per milliliter of suspension was added and the solution was spun at low speed to remove the bentonite. This method was also used where possible to free virus suspensions of ribosomes. Alternatively these particles were disrupted with Na_2EDTA-phosphate, pH 7.4. This was added to the sap to make it 10^{-2} M with respect to both Na_2EDTA and phosphate and the first high speed pellets were also suspended in 10^{-2} M Na_2EDTA–10^{-2} M phosphate. The virus was pelleted once more to remove Na_2EDTA before its affinity for bentonite was tested.

Compared with most of the other viruses used, tobacco rattle virus and potato virus X occur in low concentration in the sap. Preparation by means of differential centrifugation alone would therefore accumulate large amounts of contaminants relative to the amount of virus. For this reason, with these viruses n-butanol was used to free the sap of most of the other components. The method employed was essentially that utilized by Tomlinson and co-workers (1959) in the purification of cucumber mosaic virus. Leaves were macerated in 0.5 M phosphate buffer, pH 7.4 (50 ml per 100 g of leaf), and the sap was expressed through muslin. To the stirred ice-cold sap, n-butanol was added slowly until a concentration of 10% butanol (v/v) was reached. Fifteen minutes after the addition of the butanol had been completed, the sap was spun at 8000 rpm for 10 minutes. The clear brown supernatant was poured from the tubes and centrifuged once at high speed. After the pellets had been suspended in the appropriate salt solution and centrifuged once at low speed, the virus was ready for testing.

Examination in the analytical centrifuge showed that the virus suspensions we used were devoid of all but traces of 18 S protein and ribosomes. They were also comparatively free of green colouring matter.

Adsorption Tests

The viruses, ribosomes, and 18 S protein were tested for adsorption by bentonite in the presence of various concentrations of magnesium and sodium-potassium phosphate buffer, pH 7.4. As ribosomes are relatively labile particles, it was necessary to know that under the conditions used to test adsorption they did not disrupt. We had previously observed that they were stable in 10^{-3} M Mg^{++}–10^{-2} M phosphate and that they were destroyed when the magnesium concentration was lowered to 10^{-4} M or the phosphate concentration raised to 10^{-1} M. It was therefore clear that the relative concentrations of magnesium and other cations as well as the absolute magnesium concentration was important for the stability of these particles. Experiments showed that ribosomes remained unaltered during several hours at 5° in 10^{-4} M Mg^{++} if the phosphate concentration was only 2×10^{-4} M. It was, therefore, possible to use this low concentration of salt in the adsorption tests. The higher magnesium and phosphate concentrations employed were also found not to produce any lysis or precipitation of the ribosomes.

In most experiments the material was tested for adsorption at a concentration of 0.5–1 mg/ml using 5 mg/ml of bentonite. The concentration of most of the viruses and ribosomes was determined from measurements of the optical density at 260 mμ using appropriate extinction coefficients for the viruses (Markham, 1959), and assuming that a 1 mg/ml solution of ribosomes had an optical density of 11.1 per centimeter

(Ts'o *et al.*, 1956). The concentration of 18 S protein and of some viruses was estimated from the area enclosed by the schlieren peak seen when the material was examined in the analytical ultracentrifuge.

Tests were normally carried out in a volume of 2 ml of the appropriate salt solution. The bentonite was always added last and was rapidly dispersed by agitation of the tube. It was then immediately removed by centrifuging at 12,000 rpm for 10 minutes. The supernatants from this and a control tube containing a sample not treated with bentonite were compared in the analytical ultracentrifuge using schlieren optics. From the relative areas enclosed by the schlieren peaks, an estimate was made of the proportion adsorbed by the bentonite. Where possible a more accurate determination of the proportion adsorbed was made by measuring the optical density of the supernatant solutions at 260 mμ in a Cary spectrophotometer. After bentonite suspensions had been centrifuged in some of the salt solutions without other additions, the optical density of the supernatant was slightly higher than that of the corresponding salt solution. Allowance was made for optical density arising in this way, although it was very small except in the solution containing 10^{-4} *M* Mg^{++}. As will be discussed later, this was probably due to hydrated particles of bentonite which were not sedimented by the centrifugation used.

Ribonuclease Assay

The ribonuclease activity of sap fractions and virus preparations was tested by determining the proportion of a yeast RNA preparation rendered alcohol-soluble after several hours' incubation at 37°. Each test was carried out in a total volume of 0.5 ml. This contained 1 mg commercial yeast RNA which had been reprecipitated at 0° from *M* NaCl with 30% ethanol, and the solution was made 0.1 *M* with sodium acetate buffer, pH 5.5, this pH being optimal for leaf ribonuclease activity (Holden and Pirie, 1955b). The ribosome and soluble fractions were made from the sap of Chinese cabbage leaves. The sap was clarified by a low speed centrifugation, and the two fractions were separated by centrifuging at 45,000 rpm for 1 hour. The ribosome pellet was dissolved in the original sap volume of 10^{-3} *M* $MgSO_4$–2 × 10^{-3} *M* phosphate. The two fractions and the sap samples were diluted tenfold in 10^{-2} *M* Na_2EDTA–10^{-2} *M* phosphate buffer, pH 7.4 and these solutions used for the ribonuclease assay. In each test 0.25 ml of the diluted material was used and the samples were incubated at 37° for 4 hours. Two volumes of 90% ethanol were then added and after chilling in ice for 30 minutes, the mixture was centrifuged at 2500 rpm for 10 minutes at 0°. The supernatant was diluted tenfold with water, and the optical density at 258 mμ was measured using a 1 cm light path. Controls were made in which the diluted sap or the RNA was omitted. In one tube the RNA was hydrolyzed completely with *N* KOH at 20° for 48 hours before the addition of the ethanol, and it was by comparison with the optical density of the solution in this tube that the degree of hydrolysis of the other samples was measured.

As observed by Pirie (1956), there was much less ribonuclease in tobacco mosaic virus preparations than in sap, so that when the virus preparations were tested for ribonuclease activity, the conditions used were slightly different. The 0.5-ml samples contained 1 mg of the virus, 0.5 mg of the reprecipitated yeast RNA and the solution was made 0.2 *M* with sodium acetate buffer. The mixture was incubated for 6 hours at 37°. After the addition of two volumes of ethanol, the mixture was cooled in ice for 1 hour before centrifugation. The supernatant was diluted fivefold for optical density measurements. As shown in Table 3, the RNA in control samples without enzyme did not all precipitate with ethanol. While these controls were adequate for experiments where sap fractions were tested, it was found that the presence of tobacco mosaic virus increased the proportion of the RNA which was precipitated by ethanol in unincubated samples. In the experiments with virus, therefore, the initial proportion of ethanol-soluble RNA was determined by adding RNA to control samples of virus at

the end of the incubation immediately before the addition of the ethanol.

RESULTS

Adsorption Tests

18 S protein. From our experiments it became clear that the adsorption of ribosomes and of some viruses onto bentonite was very dependent on the magnesium concentration. The adsorption of 18 S protein, however, was much less dependent on this factor (Table 2). When tested at a concentration of 2.5 mg/ml with 1 mg/ml of 18 S protein, bentonite preparation A was estimated to adsorb 0.2 mg protein per milligram bentonite in 10^{-3} *M* $MgSO_4$–2×10^{-3} *M* phosphate. In the experiment quoted in Table 2, about 1 mg 18 S protein/ml was used so that the bentonite was probably nearly saturated with protein. Using a lower concentration of protein (about 0.7 mg/ml) resulted in almost complete adsorption at all four salt concentrations (Fig. 2). We also examined whether 18 S protein would adsorb onto sodium bentonite. The latter, prepared by treating the magnesium form with Na_2EDTA, was compared with a similar sample of the magnesium form in its capacity to adsorb 18 S protein in 10^{-4} *M* $MgSO_4$–2×10^{-4} *M* phosphate. The sodium bentonite did not adsorb a detectable amount of the 18 S protein in a test in which magnesium bentonite adsorbed 70% of the protein.

Ribosomes. When solutions containing Chinese cabbage ribosomes were treated

TABLE 2

ADSORPTION OF NORMAL PLANT CONSTITUENTS AND VIRUSES BY BENTONITE IN VARIOUS SALT MIXTURES[a]

Material	% Adsorption by bentonite in			
	10^{-4} *M* $MgSO_4$ 2×10^{-4} *M* phosphate	10^{-3} *M* $MgSO_4$ 2×10^{-3} *M* phosphate	5×10^{-3} *M* $MgSO_4$ 10^{-2} *M* phosphate	5×10^{-3} *M* $MgSO_4$ 10^{-3} *M* phosphate
18 S protein	50[b]	70[b]	90[b]	100[b]
Ribosomes	4	90[b]	100[d]	100[d]
Turnip yellow mosaic virus				
a. Cauliflower strain	0	—	0	2
b. Necrotic strain	0	—	1	1
Cocoa yellow mosaic virus	—	—	—	0[b]
Carnation mottle virus	0[b]	—	0[b]	10[b]
Sowbane mosaic virus	—	—	0[b]	—
Cowpea mosaic virus	—	—	0[b]	—
Turnip crinkle virus	2	—	11	14
Wild cucumber mosaic virus	7	10	29	92
Tobacco mosaic virus				
a. Type strain	0	3	18	66
b. Tomato mosaic virus	0	—	20	19
c. Cowpea strain (bean form)	10	—	100	100
Broad bean mottle virus	18	—	96	96
Bean pod mottle virus	—	50[b, c]	90[b]	—
Tobacco necrosis virus	30[b]	—	60[b]	80[b]
Tomato bushy stunt virus	—	90[b, c]	100[b]	—
Potato virus X	—	—	80[b]	—
Tobacco rattle virus	100[b]	—	100[b]	—

[a] Material tested at a concentration of 0.5–1.0 mg/ml with bentonite at 5 mg/ml.

[b] Estimated using the analytical ultracentrifuge; all other estimations made using the Cary spectrophotometer.

[c] Phosphate concentration 10^{-2} *M*.

[d] There was 4% residual optical density, which was shown to be due to free RNA.

with bentonite, loss of the 80 S and 125 S particles seen in the ultracentrifuge was accompanied by a corresponding loss of optical density at 260 mμ. These results could be due to an adsorption of intact ribosomes or an adsorption of at least the RNA from disrupted ribosomes. As reprecipitated yeast RNA (0.5–0.05 mg/ml) was not found to adsorb onto bentonite (1 mg/ml) under similar conditions, it seems probable that the ribosomes were adsorbed intact.

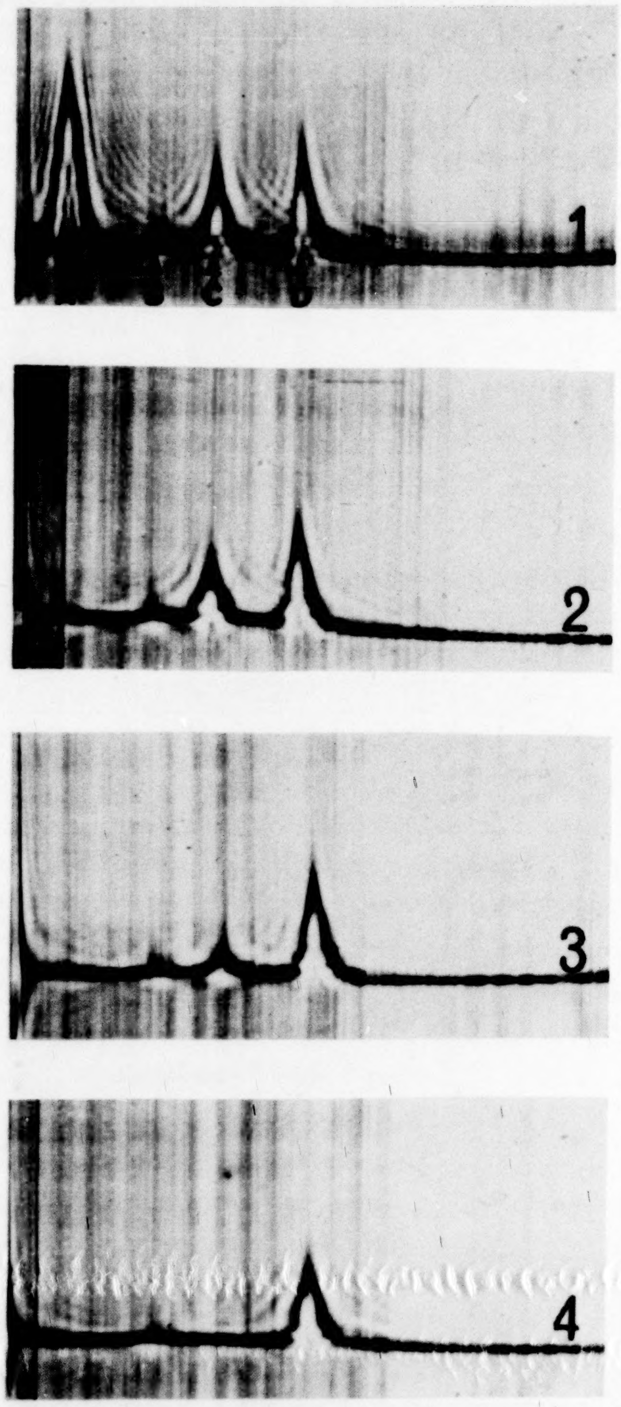

FIG. 2. Schlieren patterns of resuspended first high speed pellets from sap of Chinese cabbage plants infected with turnip yellow mosaic virus. (1) control in $10^{-4} M$ $MgSO_4$–$2 \times 10^{-4} M$ phosphate. (2–4) After treatment with 5 mg bentonite per milliliter of suspension in (2) $10^{-4} M$ $MgSO_4$–$2 \times 10^{-4} M$ phosphate, (3) $10^{-3} M$ $MgSO_4$ – $2 \times 10^{-3} M$ phosphate, (4) $5 \times 10^{-3} M$ $MgSO_4$ – $10^{-2} M$ phosphate. All photographs were taken 7 minutes after reaching speed of 35,600 rpm. *A*, 18 S protein; *B*, top component of virus; *C*, ribosomes; *D*, bottom component of virus.

As already mentioned, in $10^{-3} M$ $MgSO_4$–$2 \times 10^{-3} M$ phosphate and under conditions where about 50% of the ribosomes were adsorbed, 1 mg bentonite adsorbed 0.3 mg of ribosomes. This value applied to ribosomes from tobacco and bean leaves as well as to those from Chinese cabbage, which were mainly used to study some of the characteristics of the adsorption. As shown in Fig. 1, the amount adsorbed varied with the bentonite preparation used and the ribosome concentration. As might be expected from these results, the amount adsorbed was not directly proportional to the amount of bentonite used. In this salt mixture the capacity of bentonite for ribosomes was unchanged when the pH 7.4 phosphate buffer was replaced by one of pH 6, suggesting that near neutrality pH was not a critical factor.

The adsorption was found to be sharply influenced by the concentration of magnesium in the solution. To investigate the effect of varying the magnesium concentration on the adsorption of ribosomes and viruses, it was desirable to carry it out with little accompanying change in the amount of magnesium bound to the bentonite. As bentonite has ion exchange properties (Barr and Guth, 1951), the proportion of magnesium to other cations on the bentonite will depend on the ratio of these ions in the solution. There is also evidence that it may depend to some extent on the absolute concentration where both divalent and monovalent ions are involved (Schachtschabel, 1940). We anticipated that changes in bentonite-bound magnesium due to this latter effect would be small compared with changes due to alterations in the relative concentration of magnesium and other cations. The relative concentration was therefor kept constant in the experiments where the magnesium concentration was varied. As shown in Table 2, in the presence of $10^{-4} M$ Mg^{++}, ribosomes were only slightly adsorbed, while increasing the magnesium

concentration to 10^{-3} M resulted in considerable adsorption and complete adsorption occurred in 5×10^{-3} M Mg^{++}. This is also illustrated in Fig. 2. In another experiment we compared the adsorbing capacity of bentonite in Mg^{++} at 10^{-3} M and 5×10^{-3} M using bentonite and ribosome concentrations such that approximately 50% of the ribosomes were adsorbed. It was found using 0.5 mg ribosomes per milliliter that 0.5 mg bentonite in the solution containing 5×10^{-3} M magnesium adsorbed the same amount of ribosomes as 1 mg bentonite in the lower concentration of magnesium. The adsorbing capacity of the bentonite in the higher salt concentration was thus approximately double that in the presence of the 10^{-3} M Mg^{++} (i.e., 0.6 mg ribosomes per milligram of bentonite).

Experiments were also made in which the magnesium concentration was kept constant and the concentration of the buffer was varied. The use of a buffer concentration which was considerably lower than that of the magnesium resulted in the bentonite having a higher capacity for ribosomes. As shown in Table 2, this was also true for several viruses, which were adsorbed to a greater extent from a solution containing 5×10^{-3} M Mg^{++} when the phosphate concentration was 10^{-3} M instead of 10^{-2} M. Some of the viruses were insoluble in the lower phosphate concentrations used, so we investigated the effect of using a buffer concentration considerably higher than that of the magnesium. This change normally decreased the adsorbing capacity of the bentonite for ribosomes, but where there was a high relative proportion of ribosomes to bentonite the reverse effect was sometimes noted.

Viruses. As shown in Table 2 we tested a number of viruses for adsorption in four salt concentrations. Tomato bushy stunt and bean pod mottle viruses were found to be insoluble in concentrations of phosphate lower than 10^{-2} M. This meant that these viruses could be tested in only one of the salt mixtures normally used. They were, however, also tested using 10^{-3} M Mg^{++} in the presence of 10^{-2} M phosphate. In other instances where all four conditions were not used, it was either because of a lack of material or that the tests seemed superfluous. Our observation on the insolubility of tomato bushy stunt virus is at variance with results by other workers who found it to be soluble in pure water (Bawden and Pirie, 1938). The reason for the insolubility of our preparation is unknown, but it is possible that, as magnesium sulphate was present in the solutions used, an insoluble magnesium form of the virus was produced.

The viruses fell roughly into three groups according to their behaviour in the tests. Tobacco rattle virus resembled the 18 S protein in being readily adsorbed even in 10^{-4} M Mg^{++}. Other viruses such as tobacco mosaic virus and wild cucumber mosaic virus were only adsorbed to a great extent in the higher magnesium concentrations. Finally there were viruses like turnip yellow mosaic virus which did not adsorb appreciably in any of the solutions used (Fig. 2). With turnip yellow mosaic virus it was also shown that no adsorption occurred when 0.5 mg of the virus was treated with 17 mg bentonite in the presence of 0.13 M Mg^{++}.

Related viruses did not necessarily behave similarly. For instance, considerable adsorption of wild cucumber mosaic virus was observed in 5×10^{-3} M Mg^{++} whereas no adsorption of the serologically related (MacLeod and Markham, 1963) turnip yellow mosaic virus was noted. There was a similar difference between broad bean mottle virus and cowpea mosaic virus, which are serologically related (van Velsen and Markham, personal communication) and between the bean and tobacco forms of tobacco mosaic virus.

Preparations of turnip yellow mosaic virus and of some of the other spherical viruses (wild cucumber mosaic virus, cocoa yellow mosaic virus, cowpea mosaic virus, and bean pod mottle virus) contain two or more types of particle which sediment at different rates in the ultracentrifuge (Markham and Smith, 1949; Sinclair *et al.*, 1957; MacLeod, 1963; van Velsen and Markham, personal communication; Bancroft, 1962). The particles sedimenting fastest (bottom component) contain RNA while those sedimenting slowest (top component) may con-

sist only of protein. Where the spectrophotometer was used to measure the degree of adsorption of these viruses, the results relate almost entirely to the RNA-containing particles. However, the behaviour of the different components could be observed separately in the analytical ultracentrifuge. Observations made while purifying wild cucumber mosaic virus led us to believe that the top component of this virus was more readily adsorbed by bentonite than the bottom component. In order to confirm this we compared the degree of adsorption of the two components using the ultracentrifuge. The salt used was 10^{-3} *M* $MgSO_4$–2 × 10^{-3} *M* phosphate, as little of the bottom component was adsorbed in this solution. As shown in Fig. 3 there was no detectable adsorption of the bottom component, but approximately 50% of the top component had been adsorbed. Similar experiments on turnip yellow mosaic, cocoa yellow mosaic, and bean pod mottle viruses did not show that the different components of the same virus adsorbed differently. However, in these experiments the conditions used were such that either a substantial proportion of the virus adsorbed (bean pod mottle virus) or none was adsorbed (turnip yellow mosaic and cocoa yellow mosaic viruses). It may be

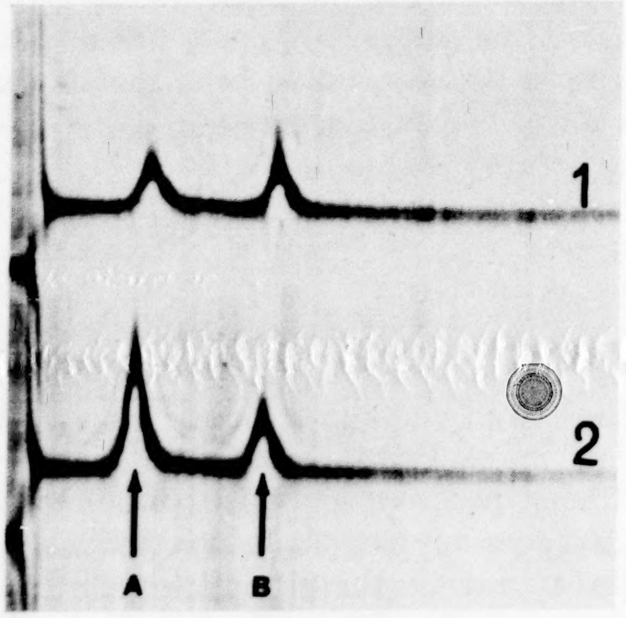

FIG. 3. Schlieren patterns of purified wild cucumber mosaic virus in 10^{-3}*M* $MgSO_4$–2 × 10^{-3}*M* phosphate. (1) After treatment with 5 mg bentonite per milliliter. (2) Untreated control. Centrifugation was at 35,600 rpm. *A*, Top component; *B* bottom component.

that under suitable conditions an adsorption differential between the different components of these viruses would also be revealed.

Preparation of Viruses Using Bentonite

The above tests indicated that under suitable conditions, it would probably be practicable to use bentonite in the purification of some of the viruses. Those viruses which showed little or no affinity for bentonite, even in the highest concentration of magnesium used, were readily purified by a straightforward use of bentonite and 5 × 10^{-3} *M* Mg^{++}. While this concentration of magnesium allowed maximum adsorption of ribosomes and 18 S protein, the use of lower concentrations of magnesium enabled us to purify a number of other viruses which would have been adsorbed to a considerable extent in the higher concentration of magnesium.

A measure of the effectiveness of these methods was obtained by making parallel control preparations using Na_2EDTA and no bentonite. A half of each infected leaf was used in making the bentonite preparation and from the other half-leaves the control preparation was made. The sap from the control half-leaves was made 10^{-2} *M* with respect to both Na_2EDTA and phosphate buffer, pH 7.4, and the medium used for resuspending the control pellets was also 10^{-2} *M* Na_2EDTA–10^{-2} *M* phosphate. The yields of virus using the two methods were compared by means of the analytical ultracentrifuge.

Where bentonite was used, an important consideration was the extent to which residual bentonite would contaminate the preparations. Such contamination might not matter if the virus was used for some purposes, but it might well give rise to error if the RNA from the virus was used for base-ratio analysis (Hitchborn and Dunn, 1963). While bentonite can be readily sedimented in high salt concentrations, hydration of the particles makes them difficult to sediment in water or at low salt concentrations (Barr and Guth, 1951). We found that the hydrated bentonite left in the supernatant after low speed centrifugation had an ultraviolet absorption spectrum with a

maximum between 240 and 250 mμ. From optical density measurements of the supernatant after centrifuging 5–10 ml of bentonite suspension (5 mg/ml) in 15-ml centrifuge tubes, it appeared that, while bentonite was completely removed by centrifuging at 12,000 rpm for 15 minutes in 5×10^{-3} *M* $MgSO_4$–10^{-2} *M* phosphate, some remained after centrifuging in 10^{-3} *M* $MgSO_4$–2×10^{-3} *M* phosphate. There were even larger amounts of unsedimented bentonite in the supernatant containing 10^{-3} *M* $MgSO_4$–10^{-2} *M* phosphate or 10^{-4} *M* $MgSO_4$–2×10^{-4} *M* phosphate. On centrifuging at 40,000 rpm for 1 hour, those supernatants which had measurable optical density gave hyaline pellets, the relative sizes of which corresponded to the relative optical densities of the supernatants. The supernatant containing 5×10^{-3} *M* $MgSO_4$–10^{-2} *M* phosphate yielded no pellets. Residual bentonite did not, therefore, present a problem in the purification of viruses using this concentration of magnesium. It would, however, be expected to contaminate viruses prepared using lower concentrations of magnesium, although as we shall show later, in the presence of sap constituents this does not always occur. The first approach we made to overcoming the difficulty of contaminating bentonite was an attempt to render all the bentonite readily sedimentable by centrifuging at 10,000 rpm instead of 12,000 rpm in its initial preparation. This procedure showed little promise so we investigated methods of removing the supernatant bentonite. Much of it could be removed from preparations of some of the smaller viruses by a low speed centrifugation of 15,000 rpm for 30 minutes. In one experiment this removed 95% of the residual bentonite while only 10% of added turnip yellow mosaic virus was lost. However, for viruses, such as tobacco mosaic virus, which have higher sedimentation constants, this method might be impracticable. An alternative approach to this problem was to reduce hydration of the bentonite by increasing the salt concentration. Experiments were made on the effect of adding various salts over a range of concentration to the supernatant from a suspension of bentonite (5 mg/ml) in 10^{-3} *M* $MgSO_4$–2×10^{-3} *M* phosphate which had been centrifuged at 12,000 rpm for 15 minutes. After the addition of the salt, the low speed centrifugation was repeated and residual bentonite was measured by optical density readings at 245 mμ. Phosphate buffer, even at a concentration of 0.5 *M* was found to be relatively ineffective in bringing about precipitation of the supernatant bentonite, whereas 0.1 *M* NaCl enabled almost all of it to be removed by the centrifugation. As expected from the relative hydration of sodium and potassium bentonite (Barr and Guth, 1951), KCl was more effective than the sodium salt and at 0.05 *M* made possible the complete removal of the bentonite. Salts of divalent and trivalent metals were even more effective, complete precipitation occurring with $MgSO_4$, $CaCl_2$, and $KAl(SO_4)_2$ at 5×10^{-3} *M*. In experiments using $MgSO_4$ the phosphate was also varied, and in contrast to the results with the total bentonite preparation, the removal of the supernatant bentonite was complete only if the phosphate concentration was not above 2×10^{-3} *M*. The effect on added tobacco mosaic virus (0.5 mg/ml) of using these salts to remove the supernatant bentonite was also tested. Use of 0.05 *M* KCl resulted in only a very small loss of virus, but addition of Mg^{++}, Ca^{++}, and Al^{+++} brought about substantial or complete loss of virus. As we shall show later, experiments with ribosomes indicate that it may be possible to use $MgSO_4$ to remove the last traces of bentonite from virus preparations if it is applied after most of the bentonite has been removed by centrifuging at 15,000 rpm for 30 minutes. Where possible it seems preferable to use this latter method as the addition of KCl may result in elution of magnesium-bound material from the bentonite.

Turnip yellow mosaic virus. The method successfully used to purify this virus was to grind every 100 g of leaf tissue with 50 ml of the bentonite preparation and 5 ml of 0.1 *M* $MgSO_4$. The sap expressed from the macerate was spun for 15 minutes at 15,000 rpm; the supernatant was poured off and spun for 60 minutes at 45,000 rpm. The pellets from this centrifugation were

suspended in $5 \times 10^{-3}\,M$ $MgSO_4$–$10^{-2}\,M$ phosphate using a volume equal to about one-tenth of that of the sap, and 0.1 ml bentonite preparation was added per milliliter of suspension. A low speed centrifugation was given to remove the bentonite and the supernatant was spun at high speed to pellet the virus. These pellets were taken up in water.

After the first cycle of centrifugation, the pellets were usually clear and, when resuspended and run in the analytical ultracentrifuge, were shown to contain the top and bottom components of the virus with very little contamination with ribosomes and other protein (Fig. 4). After the second cycle of centrifugation the virus appeared to be completely free from these constituents. Control virus prepared using Na_2EDTA was still contaminated with 18 S protein after two cycles of centrifugation, and the pellets were green. Control preparations and those made using bentonite contained virus which was closely similar in amount and in specific infectivity (using the necrotic strain). As well as being successfully applied to two strains of turnip yellow mosaic virus, the same method was used to purify carnation mottle virus and cocoa mosaic virus. With both viruses there were similar yields of virus in bentonite and control preparations, and with the carnation mottle virus the control and bentonite preparations were shown to have similar infectivities. (Carnation mottle virus was found to be insoluble in phosphate buffer at concentrations lower than $10^{-4}\,M$, so that for infectivity tests made on this virus it was dissolved in $10^{-3}\,M$ phosphate buffer, pH 7.3.) The results of the adsorption tests suggested that purification of the viruses of sowbane mosaic, turnip crinkle, and cowpea mosaic would be possible using this method.

Wild cucumber mosaic virus. While 5 mg

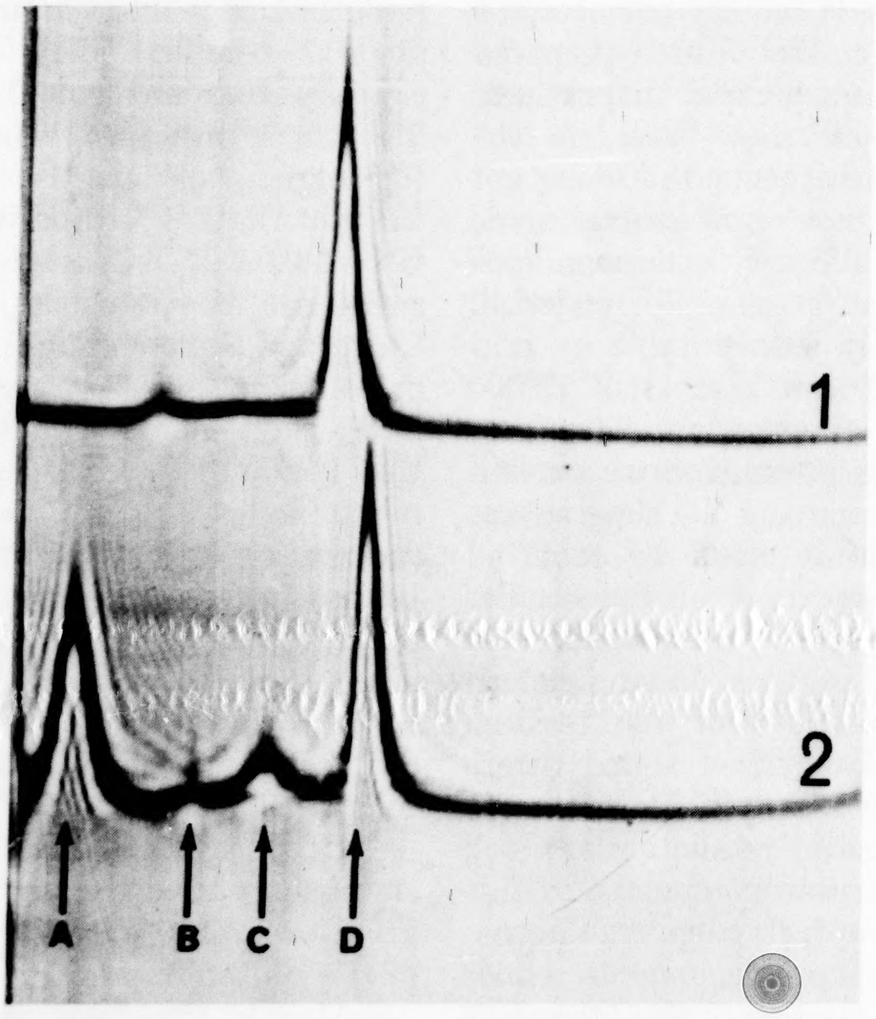

FIG. 4. Purification of turnip yellow mosaic virus with and without the use of bentonite. Schlieren patterns of resuspended first high speed pellets from sap of Chinese cabbage plants infected with turnip yellow mosaic virus. (1) Tissue treated with 25 mg bentonite per gram at time of maceration. (2) Control tissue not treated with bentonite. Centrifugation was at 35,600 rpm. *A*, 18 S protein; *B*, top component of virus; *C*, ribosomes; *D*, bottom component of virus.

bentonite per milliliter in the presence of 5×10^{-3} *M* $MgSO_4$–10^{-2} *M* phosphate and high concentrations of ribosomes and 18 S protein adsorbed little wild cucumber mosaic virus (Fig. 5), considerable adsorption of the purified virus took place under these salt conditions (Table 2). While, therefore, it was not practicable to use a high Mg^{++} concentration in the purification of this virus with the aid of bentonite, preparations could be made using 10^{-3} *M* $MgSO_4$–2×10^{-3} *M* phosphate. As pumpkin leaves give very low yields of sap, a substantial proportion of the virus can be left in the fibre. To avoid this, the salts were added in a fairly large volume; 100 g of tissue was macerated after adding 50 ml of 10^{-3} *M* $MgSO_4$–2×10^{-3} *M* phosphate and 40 ml of the bentonite preparation. After a low and high speed centrifugation, the virus, in 10^{-3} *M* $MgSO_4$–2×10^{-3} *M* phosphate, was again treated with 0.1 ml bentonite preparation per milliliter of virus suspension (as the virus pellets were found to disperse more readily in 10^{-2} *M* phosphate than in this salt at the lower concentration, the pellets were actually resuspended in 5×10^{-3} *M* $MgSO_4$–10^{-2} *M* phosphate and a fivefold dilution with water was made before the treatment with bentonite). The low and high speed centrifugations were then repeated and the virus was finally suspended in 10^{-2} *M* NaCl. This was used instead of water as this virus has been found to precipitate in the absence of salt (MacLeod and Markham, 1963). The yields of bottom component were similar in the bentonite and control preparations, but the use of bentonite reduced the yield of the top component. Some of this loss was probably due to the preferential adsorption of this component mentioned earlier, but it is also possible that in the control preparation, the 18 S protein formed a matrix which helped to retain the top component in the pellets formed on high speed centrifugation. When

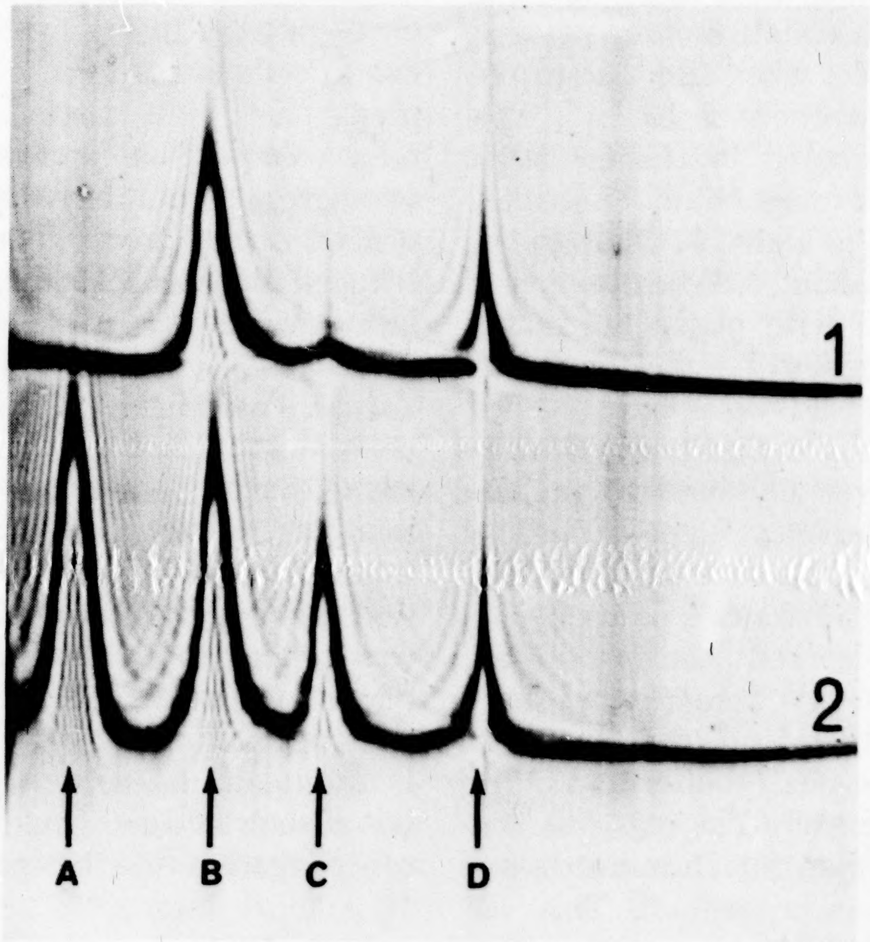

FIG. 5. The effect of treating wild cucumber mosaic virus with bentonite in the presence of 18 S protein and ribosomes. The first high speed pellets from sap of infected pumpkin were resuspended in 5×10^{-3}*M* $MgSO_4$ - 10^{-2}*M* phosphate. Shown here are the Schlieren patterns (1) after treatment with 5 mg bentonite per milliliter of suspension (2) without treatment with bentonite. Centrifugation was at 35,600 rpm. *A*, 18 S protein; *B*, top component of virus; *C*, ribosomes; *D* bottom component of virus.

the above purification procedure was carried out using healthy pumpkin leaves, the final high speed centrifugation yielded no visible pellets. From this we conclude that virus prepared in this way would not be contaminated with more than a trace of bentonite.

Tobacco mosaic virus. The data shown in Table 2 indicated that at high magnesium concentration there was considerable adsorption of tobacco mosaic virus onto bentonite. As with wild cucumber mosaic virus, however, in the presence of 10^{-3} *M* $MgSO_4$–2 × 10^{-3} *M* phosphate there was little adsorption of this virus, but both ribosomes and 18 S protein were readily adsorbed. A method for preparing tobacco mosaic virus was developed using magnesium and phosphate at approximately these concentrations. The procedure was complicated by our observation that pelleting the highly purified virus in the presence of magnesium resulted in aggregation of the particles. We found that the addition of excess Na_2EDTA to chelate the magnesium before sedimenting the virus greatly reduced the aggregation of purified virus in phosphate buffer, pH 7.4, but was without effect when added to sap to give a final concentration of 10^{-2} *M*. This difference was attributed to the fact that Na_2EDTA chelates magnesium effectively only when the pH is above 7, whereas the pH of the sap was about 6. When the pH of sap was raised to 7 with phosphate buffer, Na_2EDTA was found to be effective in reducing aggregation.

The following precedure was finally adopted, and successfully used to prepare both Type and Berkeley Type strains of tobacco mosaic virus and also tomato mosaic virus. It could not, of course, be used with the more readily adsorbed bean form of the virus. Leaves (100 g) were ground in a mortar together with 14 ml of 5 × 10^{-3} *M* $MgSO_4$–10^{-2} *M* phosphate buffer and 50 ml of bentonite suspension. The sap was centrifuged at 12,000 rpm for 15 minutes, and the supernatant was poured off. This was then made 5 × 10^{-2} *M* with respect to phosphate buffer, pH 7.4. This addition resulted in the formation of a precipitate which partially redissolved on addition of the Na_2EDTA. It was found preferable, however, to remove this precipitate by centrifuging at 12,000 rpm for 15 minutes before adding 0.5 *M* Na_2EDTA to give a final concentration of 10^{-2} *M*. The virus was sedimented by centrifuging at 36,000 rpm for 60 minutes. The whitish pellets were dissolved in water and the solution was given a low speed centrifugation at 12,000 rpm for 10 minutes. The virus was then sedimented again by repeating the high speed centrifugation. The resulting pellets were hyaline and free of 18 S protein and ribosomes, while pellets of control virus made by the same procedure omitting bentonite were still very green at this stage and contained detectable amounts of 18 S protein. The amount of virus in the bentonite preparations was only slightly less than that in the control preparations. The sedimentation rate of most of the particles in both preparations corresponded with that of the monodisperse virus, but in the bentonite preparations a small proportion (about 10%) of the material sedimented more rapidly and was assumed to be polymerized virus. The virus prepared using bentonite was slightly less infectious than the virus in the control preparation, but the difference was less than a twofold one and may have been due to aggregation. From the experiments described above in which bentonite was centrifuged alone at 12,000 rpm for 15 minutes in magnesium and phosphate concentrations similar to those used in this isolation procedure, it seemed possible that the virus pellets would be contaminated with bentonite. Such contamination does not appear to occur, however, as no pellets were obtained after the first high speed centrifugation when the same purification procedure was applied to leaves from healthy tobacco plants. If the bentonite treatment was repeated, contamination of the virus with bentonite would occur but, as has already been shown, this could be removed by centrifugation in the presence of 0.05 *M* KCl.

Use of a low concentration of magnesium. As 18 S protein and the green material from sap were still found to be adsorbed in 10^{-4} *M* $MgSO_4$–2 × 10^{-4} *M* phosphate, it seemed possible that bentonite might be used in

this salt mixture to remove such contaminants from viruses which were only slightly adsorbed at this magnesium concentration, but were readily adsorbed when the concentration was higher. Ribosomes have this type of affinity for bentonite and, because they were readily available, were used in the first experiments on purification using bentonite with 10^{-4} *M* Mg^{++}. The addition of bentonite (5 mg/ml) to sap without any other salt addition resulted in a variable loss of ribosomes, due, presumably, to variation in the concentration of divalent metal ions in the sap. The addition of the same concentration of bentonite to ribosomes resuspended after one high speed centrifugation in half the sap volume of 10^{-4} *M* $MgSO_4$–2 × 10^{-4} *M* phosphate enabled all the 18 S protein and most of the green material to be removed in a low speed centrifugation, but there was an accompanying loss of ribosomes which varied from 30 to 70%. A smaller loss of ribosomes was encountered when the bentonite treatment was applied after two cycles of low and high speed centrifugation. In order to remove most of the fine particles of hydrated bentonite, the solution was centrifuged at 15,000 rpm for 30 minutes following the addition of the bentonite. The advantage of this long low-speed centrifugation was shown by a comparison of ribosomes pelleted from this supernatant with those isolated from solutions where bentonite had been removed by centrifugation for a shorter time. Ribosome pellets from the latter contained a much higher proportion of a hyaline greenish-yellow material which presumably consisted of hydrated bentonite with adsorbed contaminants. If the supernatant from the long low-speed centrifugation was made 5 × 10^{-3} *M* with respect to $MgSO_4$ and given a further centrifugation at 15,000 rpm for 10 minutes, contamination with the greenish material was even further reduced with only about a 10% loss of the ribosomes. After this treatment the ribosomes pelleted at high speed were found to contain only very small traces of green material while control preparations made by the same method omitting bentonite were still very green. The yield was 74% of that in the control preparation. Although there was the accompanying loss of ribosomes, the treatment of the preparation with bentonite after one high speed centrifugation similarly removed most of the green material and gave high speed centrifugation pellets very similar in appearance to those obtained when bentonite was used at the later stage in the preparation. In the ultracentrifuge the bentonite preparations were found to be free of 18 S protein and consisted mainly of 80 S particles with a smaller proportion of the 125 S dimer.

Bean form of tobacco mosaic virus. Where viruses are purified using bentonite in the presence of 10^{-4} *M* Mg^{++}, the removal of ribosomes must be undertaken as a separate step. In purifying the bean form of tobacco mosaic virus, we used Na_2EDTA for this purpose. The experiments with ribosomes indicated that it would probably not be advisable to add bentonite directly to sap in purifying this virus, and we did not investigate its use in this way. A number of possible procedures were tested, and of these the following proved most satisfactory. The infected bean leaves were ground in a mortar and the sap was expressed through muslin. The fibre was washed with about 20 ml water per 100 g leaves; the washing was added to the sap, which was centrifuged at 12,000 rpm for 15 minutes. The resulting supernatant was centrifuged at 40,000 rpm for 45 minutes and the pellets were taken up in half the sap volume of 10^{-2} *M* Na_2EDTA–10^{-2} *M* phosphate, pH 7.4. The low speed and high speed centrifugation steps were then repeated and the virus pellets were taken up in half the sap volume of 10^{-4} *M* $MgSO_4$–2 × 10^{-4} *M* phosphate. Bentonite (0.1 ml/ml virus suspension) was then added and a low speed centrifugation of 12,000 rpm for 10 minutes was applied. Experiments with the tobacco form of the virus showed that when residual bentonite was removed by means of a long low-speed centrifugation there was a considerable loss of virus. Removal of bentonite was, therefore, achieved by the use of KCl. The solution was made 0.05 *M* with respect to this salt and centrifuged at 12,000 rpm for 15 minutes. This procedure removed a considerable amount of greenish yellow material, but

the virus pelleted from the supernatant by a high speed centrifugation was still contaminated by a small amount of it. This could however be completely removed if the virus pellets were allowed to resuspend in 0.05 *M* KCl and the suspension was centrifuged at 12,000 rpm for 15 minutes. High speed centrifugation of the resulting supernatant gave pellets of virus that were hyaline and only faintly brown, whereas pellets of control virus prepared by a similar method omitting bentonite were still green. When dissolved in water and examined in the analytical ultracentrifuge, the two preparations were found to contain very similar amounts of a number of components, the fastest of which had the sedimentation coefficient of the intact virus. The remainder of the peaks probably represented the shorter lengths of virus which are characteristic of preparations of this strain of the virus (Bawden, 1958). As one of the most prominent of these minor components had a sedimentation coefficient of 80 S we carried out a number of experiments to eliminate the possibility that this material consisted of ribosomes. The proportion of this component in the virus preparation was unaffected by treatment with 0.01 *M* Na_2EDTA–0.01 *M* phosphate which completely destroyed ribosomes isolated from the leaves of uninfected beans. In the ultracentrifuge the size of this 80 S schlieren peak was compared with the amount of absorption due to this component, using the ultraviolet optics. This comparison indicated that the material had too low a specific absorption to represent ribosomes. Finally, observation of the virus preparation in the electron microscope showed the presence of a high proportion of particles about 38 mμ long, which we calculate would have a sedimentation coefficient of about 80 S.

Attempts were made to reduce the number of differential centrifugation cycles necessary in purifying the virus, by destroying the ribosomes in sap using Na_2EDTA. As we have mentioned above, it was necessary to raise the pH of sap to enable Na_2EDTA to chelate effectively. This was carried out after an initial low speed centrifugation by making the sap 0.05 *M* with respect to phosphate buffer, pH 7.4, and giving a second low speed centrifugation to remove the precipitate which formed. The supernatant was then made 0.01 *M* with respect to Na_2EDTA and centrifuged at high speed. As in the preceding method, the resulting pellets were taken up in 10^{-4} *M* $MgSO_4$–2×10^{-4} *M* phosphate and treated with bentonite; residual bentonite was removed using KCl. Preparations made in this way were free of all green material, but the virus pellets were always contaminated by small amounts of buff-coloured material which was not removed by further treatment with bentonite and was difficult to remove by further differential centrifugation using water, 0.01 *M* Na_2EDTA–0.01 *M* phosphate, or 0.05 *M* KCl. The yields of virus made this way were similar to those of control preparations.

It is apparent from the foregoing experiments that whereas bentonite can be used in the presence of 10^{-4} *M* Mg^{++} to purify viruses, its usefulness under these conditions is far less than where higher magnesium concentrations can be employed. Owing to the impracticability of adding bentonite to the sap and to the need to remove fine particles of hydrated bentonite, more cycles of differential centrifugation are necessary. In addition, the removal of ribosomes must be carried out separately. However, purification of this virus was effected more rapidly with the aid of bentonite than by simple differential centrifugation. The same procedure could probably be used to purify broad bean mottle virus, and with this virus a longer centrifugation at 15,000 rpm could probably be used to remove the residual bentonite. This modification might enable the number of cycles of differential centrifugation to be reduced to three.

Elimination of Ribonuclease

Sodium bentonite is known to adsorb ribonuclease (Brownhill *et al.*, 1959; Fraenkel-Conrat *et al.*, 1961; Huppert and Pelmont, 1962), and in agreement with the observations of Petermann and Pavlovec (1963), we found that after centrifuging magnesium bentonite (25 mg per milliliter of sap) through sap from Chinese cabbage leaves, ribonuclease activity was no longer detectable (Table 3). The Table also shows

TABLE 3

RIBONUCLEASE ACTIVITY OF LEAF SAP, BENTONITE-TREATED SAP AND SAP FRACTIONS[a]

Material tested	Relative optical density of supernatant[b]		% Total RNA made alcohol soluble[c]
	Without RNA	With RNA	
Bentonite-treated sap	2	16	1
Untreated sap	4	89	72
Soluble fraction	4	79	62
Ribosome fraction	2	44	29
Control	—	13	—

[a] Each sample consisted of 0.25 ml of 0.2 *M* acetate buffer, pH 5.5, containing where appropriate 1 mg RNA and 0.25 ml of a 1:10 dilution of the sap or cellular fraction in 0.01 *M* Na_2EDTA-0.01 *M* phosphate. In the control 0.25 ml acetate buffer and 0.25 ml Na_2EDTA-phosphate only was used. After incubation for 4 hours at 37° and ethanol precipitation, the optical density of the supernatant was measured at 258 mμ.

[b] Figures given are the optical densities obtained, expressed as percentages of that obtained after KOH hydrolysis of a sample of the RNA.

[c] Allowance is made for optical density due to material tested and alcohol-soluble fraction initially present in RNA preparation.

that much of the ribonuclease activity of the sap was in the soluble fraction, a smaller proportion being associated with the ribosome fraction, as was also found by Holden and Pirie (1955a) using tobacco and pea.

The use of bentonite in the preparation of tobacco mosaic virus, as described above, also gave preparations of virus in which none of the ribonuclease activity associated with the control preparations could be detected (Table 4). Ribonuclease activity of preparations of this virus was reported by Pirie (1956), but from his work it was not clear whether the activity detected was due to contamination of the virus with a small amount of the soluble fraction from the sap or to an enzyme intimately associated with the virus. In order to distinguish between these two hypotheses, we determined the proportion of the soluble fraction that would have to contaminate the control virus to account for the ribonuclease activity detected. This was carried out by taking the sap from which the control virus had been removed by centrifugation and measuring the ribonuclease activity of this at various dilutions in the presence of a fixed amount of the bentonite-prepared virus. The presence of virus was necessary because, as already mentioned, it increased the amount of RNA precipitated in the tests and may also enhance the activity of the enzyme (Pirie, 1956). From these experiments (Table 4) we were able to conclude that the ribonuclease activity in the control virus preparation represented about 1/300 of that present in the sap. Contamination of the virus pellets with the sap supernatant should have been reduced to about 1/5000 by the isolation technique, so that much of the enzyme in the control virus preparations must be attached to the virus or to other material which sedimented with the virus. Since this work was carried out, Whitfeld

TABLE 4

RIBONUCLEASE ACTIVITY OF TOBACCO MOSAIC VIRUS PREPARATIONS AND OF SAP AT VARIOUS DILUTIONS[a]

Material tested	Relative optical density of supernatant[b]		% Total RNA made alcohol soluble[c]
	RNA added after incubation	RNA added before incubation	
Control virus	3.9	11.1	7.2
Bentonite virus (B)	2.7	2.3	−0.4
1:200 sap[d] +B	—	15.0	10.5
1:500 sap +B	—	7.3	3.9
1:1000 sap +B	—	5.6	2.6
1:5000 sap +B	—	3.8	1.1

[a] Incubation mixture contained 1 mg virus and 0.5 mg RNA in 0.5 ml of 0.2 *M* acetate buffer pH 5.5. After incubation for 6 hours at 37° and ethanol precipitation, the optical density of the supernatant was read at 258 mμ.

[b] Figures given are the optical densities expressed as percentages of that obtained after KOH hydrolysis of a sample of the yeast RNA.

[c] Allowance made for optical density observed when the RNA was added after incubation and for optical density of the diluted sap.

[d] Sap from which control virus had been removed by centrifugation. This sap was added to give the dilutions shown.

and Williams (1963) have published their more detailed investigation on this problem.

We have not investigated directly the ribonuclease activity associated with turnip yellow mosaic virus made by the bentonite method, but the RNA prepared from such virus using 40% ethanol (Markham and Smith, 1949) had sedimentation constants much higher than the 2–5 S previously reported for RNA prepared by this method (Cohen and Schachman, 1957). The RNA from the bentonite-prepared virus contained 60–85% of material which sedimented at 22 S in 0.01 *M* tris(hydroxymethyl)amino methane (Tris) buffer, pH 7.4 and was found to be infectious. After heating of the RNA in dilute solution (0.04 mg/ml) at 65° for 3 minutes, 20–30% of the RNA retained this high *S*. These preparations resembled the infectious RNA preparations made from this virus using phenol (Haselkorn, 1962). In contrast, RNA prepared by the ethanol method from control virus purified using four cycles of differential centrifugation was very polydisperse and on heating contained little or no material with an *S* of 22 S. It seems likely that control virus is contaminated with ribonuclease which degrades the RNA during isolation. When bentonite is used in the preparation of the virus, the enzyme is largely or completely removed or inactivated. Slightly higher yields of heat-stable 22 S RNA have been prepared from the virus using sodium bentonite in conjunction with the ethanol method. Full details of these experiments will be published elsewhere (Dunn and Hitchborn, in preparation).

DISCUSSION

In the purification of viruses from sap the basic demands are to remove nonvirus constituents and to concentrate the virus. The various ways in which this has been done have been reviewed by Steere (1959). Many methods involve the use of chemicals, such as alcohols or strong salt solutions, which are known to be capable of inactivating or aggregating some viruses. One of the gentlest methods, and one that is now widely used, employs differential centrifugation. Several cycles of low speed and high speed centrifugation are usually needed. As we have shown, the use of bentonite with this method can considerably reduce the number of centrifugation cycles necessary to obtain virus preparations of a purity comparable to that of preparations made without the use of bentonite. Moreover, the supernatants from the low speed centrifugations can be poured off without causing any disturbance of the pellets, which can seldom be done in the absence of bentonite. Where differential centrifugation is used alone, work in this laboratory has shown that it is usually advantageous to leave the pellets of virus to stand for several hours in the resuspending medium, so that the virus may redissolve without much dispersion of the associated green material. Where bentonite is employed, there is no disadvantage in rapidly homogenizing the green pellets.

Some viruses, such as those of brome mosaic, wound tumour (Brakke, 1963), and potato yellow dwarf (Brakke, 1956) require magnesium or other divalent cations for their stability. In their preparation, therefore, it is unlikely that Na_2EDTA or other chelating agents could be used to remove ribosomes. It is possible, however, that under suitable conditions, bentonite could be used to eliminate ribosomes from preparations of these viruses.

A possibly more important function of bentonite in the preparation of viruses is the removal of ribonuclease or the inhibition of its activity. While little work has been devoted to the ribonuclease contamination of plant viruses, the preparation of infectious RNA from them has only been achieved by using treatments which rapidly remove, inactivate or inhibit ribonuclease (Gierer and Schramm, 1956; Fraenkel-Conrat, 1956; Kaper and Steere, 1959; Reichmann and Stace-Smith, 1959). Even with these methods, a high proportion of the extracted RNA is frequently of low molecular weight and the infectivity of the product is often lost on storage. Modifications of these methods which further reduce ribonuclease activity have been shown to improve the results obtained (Singer and Fraenkel-Conrat, 1961; Kodama and Bancroft, 1964). Reduction in the initial contamination of

the virus with ribonuclease may similarly help to improve the yield and stability of the infectious RNA. As we have shown with turnip yellow mosaic virus the use of bentonite in the preparation of the virus reduces contamination with ribonuclease sufficiently to enable infectious RNA to be isolated from the virus by use of 40% ethanol.

Many viruses lose infectivity fairly quickly *in vitro*, and it has been suggested (Haselkorn, 1962) that this may be due to the activity of ribonuclease in the preparations. If this is true, then preparations of these viruses which are free of this enzyme should remain infectious for longer periods. Experiments to test this possibility are in progress.

There are at least three different ways in which the contamination of preparations with ribonuclease can arise. As we have shown, much of the enzyme in sap is present in the nonsedimenting fraction. It would seem inevitable that some of this would contaminate the virus pellets where only a few cycles of differential centrifugation were given. However, our studies and those of Whitfeld and Williams (1963) indicate that most of the contamination in tobacco mosaic virus is not from this source. The second possibility is that the enzyme is on particles which are sedimented along with the virus. The work of Pirie (1956) suggests that there can be contamination of this type when ribosomes are present in the virus preparation but it seems from the work of Whitfeld and Williams (1963) that other contaminating particles may also be involved. The use of bentonite at any stage in the preparation would be expected to remove the enzyme in free solution and that present on the ribosomes. While we have not examined the possibility very carefully, it seems likely that bentonite also removes the ribonuclease-containing material described by Whitfeld and Williams (1963). This we conclude from the fact that while these authors obtained small pellets of this material from leaves of healthy tobacco plants put through the process they used for isolating the virus, in our experiments no pellets were found when similar leaves were subjected to the bentonite procedure. The third possibility is that the enzyme is actually adsorbed onto the virus particles. Such contamination may occur in the intact cell, and if so, its prevention presents a problem outside the scope of the present work. It is possible, however, that ribonuclease becomes adsorbed onto the virus during the isolation procedure. Initially we considered that such contamination was most likely to arise from enzyme released when the ribosomes were disrupted during the isolation of the virus. This view was supported by studies on *Escherichia coli* and yeast (Bolton *et al.*, 1959; Tal and Elson, 1963; Ohtaka and Uchida, 1963) which showed that most or all of the ribonuclease activity in extracts of these organisms was present in the ribosomes. As already mentioned, however, experiments showed that in the sap from Chinese cabbage leaves, there was more ribonuclease activity associated with the soluble fraction than with the ribosome fraction. This was in agreement with the earlier work of Holden and Pirie (1955a) on the ribonuclease activity of fractions of sap from leaves of tobacco and pea. It followed from this observation that the removal of the ribosomes intact was only of minor significance in avoiding adsorption of ribonuclease onto the virus. However, the ribonuclease found free in extracted sap may, in the living cell be associated with particles, other than ribosomes, which are unstable and are destroyed when the cell structure is disorganized by grinding. The association of ribonuclease with such unstable particles has been demonstrated in animal tissues (de Duve *et al.*, 1955) and similar particles could be present in leaf cells. If this is so, then the probability of virus adsorbing ribonuclease would be reduced by having bentonite present when the leaves are ground. We have applied this principle wherever possible in the methods developed for purifying viruses with the aid of bentonite. Further work will be necessary to determine whether our methods do, in fact, enable virus to be prepared which is completely free from ribonuclease.

The viruses tested included representatives of two major morphological types, namely those with rod-shaped particles

and those having approximately spherical particles. There was great variation in affinity for bentonite and not only did we find no correlation between the particle shape and the readiness to adsorb, but closely related viruses behaved very differently. Two examples of this were the bean and tobacco forms of tobacco mosaic virus, and cowpea mosaic virus and the serologically related bean pod mottle virus. This type of difference may also apply to two strains of tobacco necrosis virus which have been shown to differ in the extent to which they lose infectivity on treatment with bentonite (Kassanis and Welkie, 1963). Viruses can be contaminated with adsorbed proteins and other substances (Markham, 1959), and it is just possible that these could be responsible for the differences in adsorption between viruses or strains. While such a hypothesis has some substance where the viruses had been isolated from different host plants, it can hardly explain the difference we found between the two components of wild cucumber mosaic virus. Moreover, as the two components have the same amino acid composition (Symons *et al.*, 1963) the difference in adsorption between them presumably depends on a very subtle difference between the proteins.

Our experiments showed that the adsorption of ribosomes, 18 S protein and many of the viruses depended on the presence of magnesium. 18 S protein, which adsorbed readily onto magnesium bentonite, showed no detectable adsorption onto the clay in the sodium form. While the same probably applies to ribosomes and those viruses which showed a lower affinity for bentonite when the magnesium concentration was reduced, it may not be true of viruses such as tobacco rattle virus which did not show this trend in affinity. These may adsorb onto sodium bentonite.

Where adsorption depended on the magnesium concentration, then, in general, either an increase in the magnesium concentration or a decrease in the concentration of monovalent cations resulted in increased adsorption and vice versa. Bentonite, however, has ion-exchange properties and as a result can modify the salt conditions, especially where the relative concentrations of magnesium and other cations are very different from those in which the bentonite was prepared. We believe that an exchange of this type was responsible for the anomalous result obtained with ribosomes, in the presence of a high proportion of bentonite, where an increase in the buffer concentration from $10^{-3}\,M$ to $10^{-2}\,M$ increased instead of decreasing the adsorption of ribosomes.

The most likely explanation of magnesium-dependent adsorption onto bentonite is that magnesium ions are needed to link protein to bentonite. The large differences between proteins in their affinity for bentonite may then be due to differences between the amounts of magnesium bound to the surfaces of different viruses. That turnip yellow mosaic virus can bind substantial amounts of magnesium (Johnson, 1964) and, as we have shown, not possess an affinity for bentonite does not necessarily contradict this hypothesis, for much of such bound magnesium may be present inside the virus particles on either the RNA (Loring and Waritz, 1957) or the protein.

While we have mainly concerned ourselves with the use of magnesium bentonite in the purification of plant viruses, its property of selectively adsorbing proteins may be of use in the separation of virus strains, in the purification of some animal viruses and in related fields of research. We have already described its use in the purification of ribosomes, but it seems likely that there is a possibly more important application in the preparation of sRNA. Preliminary experiments indicate that, by using bentonite, sRNA can be prepared from leaf sap in good yield without the use of the ultracentrifuge (Dunn, unpublished), and it is probable that the method will be applicable to a wide range of organisms.

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The Uptake and Development of Reovirus in Strain L Cells Followed with Labeled Viral Ribonucleic Acid and Ferritin-Antibody Conjugates[1]

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Reovirus possesses an outer capsid and a subjacent shell, presumably necessitating the rupture of two separate protective coats to release the genome. This may occur intracellularly within phagocytic viral inclusions where whole particles are sequestered and gradually lose their coats. Cells infected with purified virus particles containing labeled RNA were sampled at intervals during a 12-hour experimental period, and then subjected to light and electron microscopic autoradiography. The label was conserved in a macromolecular form throughout this period. Because of asynchrony in the release of parental genomes and lack of identifiable, partially degraded forms of the virus outside the phagocytic inclusions, it was not possible to reconstruct the sequence of stages during the penetration of inoculum RNA to its site of function. However, about 14% of the parental label was finally transferred to foci where progeny virus was being formed. Evidence, derived from experiments with infected cells and tagging by means of ferritin-antibody conjugates specific for reovirus protein, is presented which relates virus morphogenesis with the presence of mitotic-spindle tubules and a cytoplasmic filamentous component.

INTRODUCTION

This study, one of a series dealing with the penetration of animal viruses as it is related to the release of their nucleic acids and initiation of biosynthetic functions, concerns the early events in the association of reovirus with L cells. Reoviruses have a relatively large size of 700 A, possess a characteristic icosahedral shape, contain RNA of high molecular weight (Gomatos and Tamm, 1963), and have a unique affinity for tubules of the mitotic spindle (Dales, 1963a). These properties have made it possible to identify by autoradiography the RNA of inoculum particles labeled with radioactive tracers and to follow in time the intracellular fate of this RNA. Ferritin-conjugated antibody (Hsu *et al.*, 1963) was used to identify intracellular protein of the viral progeny.

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MATERIALS AND METHODS

Culture Medium

The basic medium used in all experiments was Eagle's MEM (Eagle, 1959) supplemented with 5% fetal calf serum (Microbiological Associates).

Cells and Virus

Strain L 929 cells were cultured in roller tubes (Siminovitch *et al.*, 1957). Stocks of purified reovirus type 3, Dearing strain, were prepared in a manner described pre-

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Purification of Barley Yellow Dwarf Virus[1]

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A vector-specific strain of barley yellow dwarf virus (transmitted selectively by *Macrosiphum avenae*) was partially purified from frozen oats by differential centrifugation following clarification by chloroform. Since the virus is not mechanically transmissible to plants, infectivity assays were based on feeding aphid vectors through membranes on test inocula. Concentrated virus preparations stored at 3°C remained infectious for up to 16 weeks; similar preparations were infectious following treatment (18 hours at 15°C) with any of several proteases and nucleases. A major limiting factor in purification appeared to be the low concentration of virus in source plants; usually the yield of purified virus from 1 liter of plant juice was only about 25–50 μg. Analytical sucrose density-gradient centrifugation of preparations made from infected oats revealed a component (sedimentation coefficient 115–118 S) that was not present in comparable preparations made from healthy oats in each of three experiments. A dense polyhedral particle about 30 mμ in diameter was the major constituent of the extra component from infected plants. This polyhedral particle is believed to be the virus because it sedimented to the same depth as did the infectious entity in rate- and equilibrium-zonal centrifugations, and it migrated with the infectious entity in Sephadex[4] gel filtration. The particle also was obtained from viruliferous aphids.

INTRODUCTION

Many detailed studies have been made on the properties of some plant viruses, but virtually nothing is known about the nature and properties of some other plant viruses.

Barley yellow dwarf virus (BYDV) is one of a group of aphid-transmitted plant viruses whose nature and properties are unknown. Unlike most aphid-transmitted viruses, BYDV has not been transmitted mechanically to plants, and the virus seems to be restricted to phloem tissue of infected plants. After an aphid vector has fed on infected plants it is able to transmit BYDV for many days, often as long as the insect lives. Among viruses of this group, the persistent or circulative aphid-transmitted viruses, potato leaf roll virus has received most attention; progress in attempts to purify it generally has been slow and difficult (Day and Zaitlin, 1959; Heinze, 1955; Stegwee and Peters, 1961).

A serious handicap in studies on purifica-

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[4] Mention of specific equipment, trade products, or a commercial company does not constitute its endorsement by the United States Government over similar products or companies not named.

tion of viruses like potato leaf roll and barley yellow dwarf is the difficulty of making infectivity assays because the viruses cannot be transmitted to plants mechanically. This difficulty can be partly overcome in studies on BYDV because two different methods can be used to test infectivity of virus preparations by using aphids as vectors. One assay method is based on acquisition of virus from liquid preparations by aphids feeding through membranes; the other is based on mechanical transmission of virus to aphids with fine glass needles. Both techniques are somewhat tedious and laborious, but they do make studies on purification of BYDV possible.

This paper reports results of studies on purification and properties of BYDV and on the relation between virus infectivity and a polyhedral particle observed in purified preparations.

MATERIALS AND METHODS

General

Stock colonies of virus-free aphids were maintained on caged barley plants (*Hordeum vulgare* L.) under precautions previously described (Rochow, 1959). Aphid colonies usually were grown under continuous fluorescent illumination at about 70°F. Most aphid colonies were started from 10 viviparous females that came from a special colony started from newly emerged nymphs. Until recently, *Macrosiphum avenae* (Fabricius), the English grain aphid, had been referred to as *M. granarium* (Kirby). At least 30 aphids from each group used were always tested as controls in each experiment; in all cases the aphids proved to be free of BYDV.

All experiments were done with a vector-specific strain of BYDV that is transmitted selectively by *M. avenae*; it originally was obtained from an infected oat plant in New York (Rochow, 1959). The virus was maintained by serial transmission to new oat plants at intervals of 5–6 weeks. Comparative tests with 4 aphid species at each transfer showed that the relative vector specificity of the isolate remained unchanged throughout the studies (Rochow, 1963a, b). Coast Black or California Red oats (*Avena byzantina* C. Koch) were used to maintain the virus, to make infectivity tests, and to provide a source of infected material for most purification studies. Plants were grown in 4-inch clay pots of composted, steam-sterilized soil.

Infectivity of BYDV preparations was estimated by a membrane-feeding method (Rochow, 1960; Pang and Rochow, 1963). Virus-free *M. avenae*, previously starved at 15° for 6–8 hours, were placed in small feeding chambers topped with stretched Parafilm M[4]. Test inocula were placed on the opposite side of the membrane so that aphids could feed through the membrane on the liquid preparations. Inocula usually were diluted in 20% sucrose dissolved in neutral 0.1 *M* potassium phosphate buffer. Following an acquisition feeding period of about 18 hours at 15°, aphids were transferred to oat seedlings (10 aphids per plant) for a 5-day inoculation test feeding period. Aphids were then removed by fumigation with lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane) or DDVP (*O,O*-dimethyl 2,2-dichlorovinyl phosphate) in a closed chamber, and the plants were placed on a greenhouse bench under supplemental illumination, where they were observed weekly for at least 4 weeks. In each experiment aphids that had not fed on the virus preparations were also placed on test plants to serve as controls.

In some experiments infectivity assays were also made by a needle-injection method (Mueller and Rochow, 1961). Aphids injected by means of fine glass needles were placed, usually 5 injected aphids per plant, on oat seedlings for an inoculation test feeding period of 5 days.

Purification Procedure

BYDV-infected oats were the source for most purifications. Infected plants were obtained by allowing groups of viruliferous aphids to feed for 3–5 days on oat seedlings. Following this inoculation test feeding period, plants were fumigated and placed on a greenhouse bench under supplemental illumination for 6–10 weeks. Pots were occasionally given supplemental fertilization with a commercial 5–10–5 fertilizer. Entire shoots were harvested, chopped into pieces

about 5 cm long, and stored at about −20°C in plastic bags. Infective preparations have been made from tissue stored for several years, but the highest yields usually were obtained from tissue stored 1–12 months. Healthy oats used to prepare control preparations were treated in the same manner except that they were not infested with aphids and were not always stored in the freezer for the same periods as the infected tissue.

Frozen source tissue was partially thawed at the start of each purification experiment. The tissue was then ground in a type D fruit juice extractor attachment for a Hobart[4] meat chopper. To each 9 ml of cold crude juice (pH ~6.3), 1 ml of neutral 1.0 *M* potassium phosphate buffer was added. Three milliliters of chloroform and 1.5 ml of *n*-amyl alcohol were then added to each 9 ml of adjusted (pH ~6.8) crude juice (Schneider, 1953). The mixture was stirred vigorously by a magnetic stirrer for 15 minutes at room temperature and then chilled in an ice bath. The emulsion was broken by centrifugation for 10–20 minutes in an International[4] Clinical centrifuge, usually at 4°. The aqueous layer was collected and centrifuged again for 20 minutes at 4800 rpm in a Servall SP[4] centrifuge at 4° to produce clarified juice. The clarified juice was next centrifuged for 4 hours at 29,000 rpm in the no. 30 rotor of the model L[4] Spinco centrifuge. Pellets were usually soaked overnight in 0.1 *M* phosphate buffer at 4° (usually about 2 ml buffer per pellet) and resuspended by means of a stirring rod and ground glass homogenizer. The concentrated preparation was then centrifuged for 20–30 minutes in the Servall centrifuge at 4°. The virus was again pelleted by centrifugation in the Spinco centrifuge and resuspended in 0.1 *M* potassium phosphate buffer, usually 1 ml per 100 ml of clarified juice. Final preparations were centrifuged for 20–30 minutes in the Servall centrifuge at 4°. When preparations more concentrated than 100-fold were made, the procedure usually was carried through an additional cycle of differential centrifugation.

Density-Gradient Centrifugation

Density-gradient centrifugation was carried out in a model L Spinco centrifuge, usually at about 9° (Brakke, 1960). Gradient columns for rate-zonal centrifugation were made by layering 4, 7, 7, and 7 ml of solutions containing 100, 200, 300, and 400 mg sucrose per milliliter, respectively, in plastic centrifuge tubes. Gradient columns for equilibrium-zonal centrifugation were prepared by layering 0.9 ml each of solutions containing 300, 400, 500, and 600 mg sucrose per milliliter. Unless stated otherwise, the sucrose was dissolved in 0.1 *M* neutral potassium phosphate buffer. Tubes were stored at 4° usually for 2 days before use. Rate-zonal centrifugations in the SW-25 rotor were at 23,000 rpm, usually for 3 hours. Equilibrium-zonal centrifugations in the SW-39 rotor were for 5 hours at 36,000 rpm. One milliliter of concentrated preparation was floated on each gradient tube. Samples, usually 1.0 ml, were removed from the gradient columns by puncturing the plastic tube with a needle attached to a hypodermic syringe. About 0.2 ml of each sample was placed in a small plastic centrifuge tube, wrapped in Parafilm, and packed in an insulated container together with a can of ice water. The package was then sent by air mail, special delivery, from Ithaca to Lincoln. In general, packages taken to the Ithaca Post Office at 11:00 A.M. reached the laboratory in Lincoln early the following morning.

For analytical density-gradient centrifugation, centrifuged rate-zonal gradient columns were photometrically scanned with the ISCO Density Gradient Fractionator[4] (Brakke, 1963) using the sensitive (0.5 O.D.) scale and an external recorder. Five inches on the external recorder chart equaled 0.25 absorbancy units at 253.6 mμ. Used in this way, the ISCO scanner will detect 1 μg of virus. At least 5–10 μg of BYDV was required to produce a zone visible by scattered light.

Deviations from the above procedures are noted in the descriptions of results of specific experiments.

Electron Microscopy

The concentration of virus was too low to give a countable number of particles on

specimen grids on which dialyzed samples from gradient columns were sprayed from low-pressure Vaponefrin[4] atomizers. Suitable concentrations of particles were obtained on carbon-backed, collodion-coated specimen grids by a modification of the method of Vatter (Black *et al.*, 1963). A large drop of the sample in sucrose solution was allowed to stand on the grid for 3 hours at 2°. The drop was large enough so that its edge was not observed in the microscope. The number of particles at the edge of a droplet was disproportionately large. Excess liquid was removed with a piece of filter paper and the grid was floated specimen-side down on 20% glycerin for 15 minutes at room temperature to remove the sucrose. Washing grids on water removed more virus than did washing on 20% glycerin. Most excess glycerin was removed with filter paper and the specimen was either shadowed or positively stained with uranyl acetate. For positive staining, a drop of 2% uranyl acetate was placed on the grid for 45 seconds, removed with filter paper, and the specimen was then washed for 1 minute on 20% glycerin.

Specimens were either shadowed from one direction or very lightly shadowed from three directions 120 degrees apart, with tungsten oxide. In the latter case, the filament was 3 cm higher than the specimen and 17 cm horizontally away from it. Tungsten oxide was evaporated from each of the three directions until deposited metal was just visible behind a machine nut on white tape 1.5 cm from the filament.

Specimens were examined in either a RCA EMU 2D or EMU 3G electron microscope.[4] Five 2 x 2-inch micrographs at 15,000 × were made of randomly selected areas from two grids for each sample. Areas were selected adjacent to dirt suitable for focusing, not on the basis of particles suspected of being virus. Particles were counted on the negatives under a stereoscopic microscope at 10 ×. In some experiments, relative numbers of particles were counted directly on the microscope screen at 105,000 ×, as noted in footnotes to Tables 4 and 5.

All methods, except the spray-droplet methods, proposed for quantitative electron microscopy involve washing of specimens on the grid, during which particles may wash off. The present electron microscope methods share this fault. Nevertheless, the number of particles was inversely proportional to the dilution of the virus preparation. Apparently a certain fraction of particles colliding with the collodion adhere to it. Since the number of collisions is proportional to the concentration of particles in solution, the number of particles adhering to the membrane should be, and was, proportional to the concentration. At high concentrations, of either virus particles or impurities, when the membrane became covered, this proportionality did not hold.

RESULTS

Stability of BYDV

Several lines of evidence suggested that stability was not a major limiting factor in purification of BYDV. Partially purified preparations made from frozen, chloroform-treated material were infectious; similar preparations stored at 4° remained infectious for many weeks, and infectivity was not destroyed in partially purified preparations treated with proteases, nucleases, or mixtures of the enzymes.

Since preliminary experiments had shown the feasibility of using frozen tissue as a source of virus (Rochow, 1960), all experiments were carried out with plant tissue that had been stored at about −20°. Careful comparisons among different clarification procedures have not been made because results with the chloroform procedure generally have been satisfactory. Original indications of loss of virus by treatment with chloroform (Rochow, 1960) were not substantiated in other experiments. In one test of clarification by chloroform, for example, juice from 2486 g of infected oats was divided into two lots. A partially purified preparation concentrated 30-fold relative to the original amount of juice was prepared following clarification by chloroform. The second lot of juice was used to make a parallel preparation following clarification only by low-speed centrifugation for 30 minutes; this preparation was very green in color compared with the chloroform-clarified one. The relative amount of BYDV in each preparation was estimated by

both the membrane-feeding and the needle-injection assays. Aphids that fed through membranes on samples of the preparation originally clarified only by centrifugation transmitted virus to 5 of 15, 2 of 15, and 2 of 15 plants following feeding on dilutions of 1:1, 1:10, and 1:100, respectively. Aphids that fed on corresponding dilutions of the chloroform-clarified preparation transmitted virus to 10 of 15, 9 of 15, and 4 of 15 plants, respectively. None of 15 plants infested with control aphids became infected. Aphids injected with a 1:1 (undiluted) or a 1:100 dilution of the preparation clarified only by centrifugation transmitted virus to 4 of 4 and 0 of 4 plants, respectively. Similar groups (5 injected *M. avenae* per plant) of aphids injected with corresponding dilutions of the chloroform-treated preparation transmitted virus to 6 of 6 and 4 of 8 plants, respectively. None of 40 aphids tested as controls in the injection assay transmitted virus.

BYDV appears to be stable in partially purified preparations stored in the cold. In one experiment on stability during storage, 3 different solvents were used to prepare comparable virus preparations concentrated 2-fold. Membrane assays were made immediately after the concentrated preparations were prepared, and at intervals of 5, 12, and 19 days of storage at 3°. Results (Table 1) indicated no major loss of infectivity during storage and suggested the use of 0.1 *M* neutral potassium phosphate buffer as solvent. Preparations stored for periods longer than 19 days also remained infectious. On one occasion, for example, infectivity was detected (5 of 12 plants infected) in a partially purified virus preparation that had been stored for 4 months at 3°. Electron microscopy indicated that the particle described below was morphologically intact after at least one month's storage of a purified preparation at 2°.

Another indication of the stability of BYDV was obtained in enzyme treatments carried out on partially purified preparations in cooperation with Dr. Robert W. Holley (Department of Biochemistry, Cornell University). When preparations were incubated for 18 hours at 15° with chymotrypsin, trypsin, ribonuclease, deoxyribonuclease, or combinations of the enzymes, infectivity of treated preparations was essentially the same as that of control preparations in assays made by membrane-feeding or by needle-injection.

Correlation of Particle and Infectivity

Density-gradient centrifugation. Although BYDV appeared to be reasonably stable and could be concentrated by differential centrifugation, early attempts to study the nature of the infectious particle were hampered by the apparent low titer of virus in concentrated preparations. Virus preparations concentrated 15- to 70-fold were always infectious, but a visible zone was not consistently associated with infectivity in density-gradient centrifugation. Moreover, infectivity of zones removed from density-gradient tubes was not related to absorbance

TABLE 1

INFECTIVITY OF BARLEY YELLOW DWARF VIRUS AFTER STORAGE AT 3°C IN DIFFERENT SOLVENTS

Solvent	Infectivity of sample stored for number of days indicated[a]			
	0	5	12	19
Distilled water	1/18	2/18	1/18	2/18
0.1 *M* potassium phosphate buffer, pH 7.0	10/18	11/18	16/18	13/18
Juice from healthy oats	5/18	6/18	7/18	6/18
Aphid control	0/18	0/18	0/18	0/18

[a] Number of plants that became infected over number of plants infested with 10 aphids (*M. avenae*) that had fed through membranes on test sample. For assay, 2 ml of 40% sucrose was added to 6 ml of each sample, which had been pelleted by centrifugation and resuspended in ½ volume of the solvent shown.

at 260 and 280 mμ as measured in a Beckman DB spectrophotometer.[4] When increased sensitivity of the ISCO density-gradient fractionator and scanner made it possible to detect as little as 1 μg of virus in zones following density-gradient centrifugation, concentrated preparations of BYDV and comparable preparations from healthy oats shipped from Ithaca to Lincoln were examined by means of this instrument following density-gradient centrifugation.

Concentrated preparations made from infected oat plants by chloroform treatment and differential centrifugation had an extra component, not present in similar preparations from uninfected oats, when analyzed by density-gradient centrifugation (Fig. 1). This extra zone was often too faint to be visible by scattered light. About 98% of the light-absorbing components in the partly purified preparations remained near the meniscus during density-gradient centrifugation. The extra component in the extracts from diseased plants accounted for 2% of the light absorption. If the extra component has the specific extinction coefficient of southern bean mosaic virus, about 25–50 μg of the component was isolated per liter of clarified oat juice.

The extra component was detected after density-gradient centrifugation in columns with the sucrose dissolved in 0.01 *M* Tris-HCl [tris(hydroxymethyl)aminomethane], pH 9.0; 0.05 *M* sodium borate, pH 8.0; 0.01 *M* potassium phosphate, pH 6.0; and 0.01 or 0.1 *M* phosphate, pH 7.0 (Fig. 1). The virus apparently degraded in glycerin because only a very small peak was observed after density-gradient centrifugation through columns prepared from 4, 7, 7, and 7 ml of 10, 20, 30, and 40% (v/v) of glycerin. In other tests using glycerin columns, however, active virus was recovered from the tubes.

A mixture of approximately 50 μg each of purified southern bean mosaic and tobacco mosaic viruses and 25 μg of purified brome mosaic virus was centrifuged through one gradient column while samples of partly purified, concentrated preparations from diseased and healthy oat plants were centrifuged through columns in the other two tubes of the rotor. The extra component from the tube containing BYDV sedimented at almost exactly the same rate (or a few per cent faster), as did southern bean mosaic virus (Fig. 1). Since the sedimentation coefficient of southern bean mosaic virus is 115 S (Miller and Price, 1946), that of BYDV must be 115–118 S.

The extra component was observed by

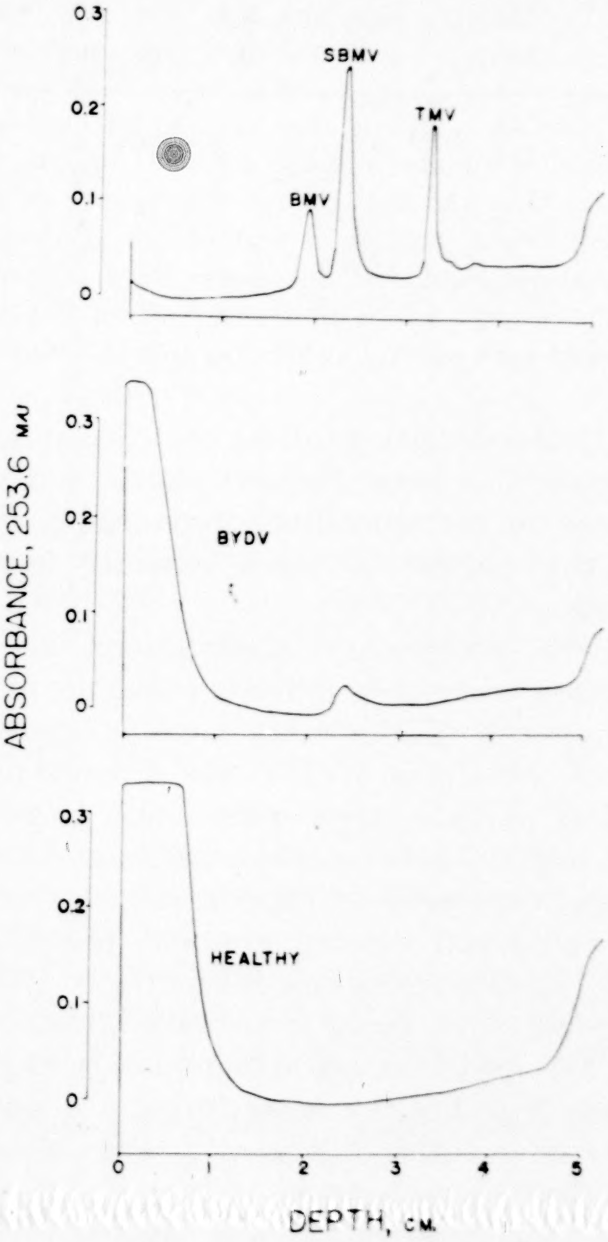

FIG. 1. Scanning patterns of gradient columns centrifuged after the addition of brome mosaic virus (BMV), southern bean mosaic virus (SBMV), and tobacco mosaic virus (TMV) as markers (top); or after addition of 0.2 ml of a partly purified preparation of barley yellow dwarf virus (BYDV) concentrated 500 × (i.e., 1 ml from 500 ml clarified juice) (middle); or after addition of a preparation concentrated 500 × made from healthy oats (bottom). Centrifugation was for 3 hours at 23,000 rpm; sucrose was dissolved in 0.1 *M* potassium phosphate buffer, pH 7.0. The area under the BYDV peak is that expected for 3.75 μg of southern bean mosaic virus.

TABLE 2

RELATIVE CONCENTRATIONS AND INFECTIVITIES OF PREPARATIONS USED IN RATE-ZONAL DENSITY GRADIENT EXPERIMENTS

Expt. no.	Relative concen-tration	Infectivity of successive dilutions of sample[a]
1	100×	8/9, 9/9, 7/9, 3/9
2	100×	9/9, 2/9, 0/9, 0/9
3	500×	8/9, 9/9, 8/9, 6/9, 0/9, 1/9
4	180×	9/9, 9/9, 9/9, 8/9, 2/9

[a] Number of plants that became infected over number of plants infested with 10 aphids (*M. avenae*) that had fed through membranes on test sample. First fraction is test of 1:5 dilution of preparation indicated; successive fractions represent successive 5-fold dilutions. None of 36 plants infested with control aphids became infected.

analytical density-gradient centrifugation in preparations from diseased plants, but was absent in corresponding preparations from healthy plants, in three separate experiments.

Since preliminary observations in the electron microscope indicated that the major component of the extra zone in gradient tubes containing BYDV was a dense polyhedral particle, tests were made to study the relation between the particle and infectivity. One series of experiments was based on rate-zonal density gradient centrifugation. Four preparations concentrated 100- to 500-fold were made from oats infected by BYDV, and 4 comparable preparations were made from healthy oats, during a period of several months (Table 2). For density-gradient centrifugation at Ithaca, 1.0 ml of the virus preparation was layered on one tube, 1.0 ml of the preparation from healthy oats was layered on a second tube, and 1.0 ml of a purified preparation of southern bean mosaic virus (containing 0.1 mg of virus) was layered on a third tube. Following centrifugation at about 9° in the SW-25 rotor for 3 hours at 23,000 rpm, the tubes were examined in a beam of light in a dark cold room. A visible zone was observed in the tube containing BYDV 1.6 cm below the meniscus in experiments 3 and 4 but not in 1 and 2 (Tables 2 and 3). A zone was never observed in this location in any tube containing material from healthy oats. When the zone was visible in the BYDV tube, it was always at the same location as the zone in the "marker" tube containing southern bean mosaic virus. When a visible zone was not observed for BYDV, the location of the southern bean mosaic virus zone was used as a basis for removing samples from the BYDV "zone." Three samples were removed from each tube containing BYDV. One sample was from 0.5 cm above the "virus zone," a second sample was from the "virus zone," and a third sample was from 1.0 cm below the zone. In each experiment, a sample was also removed from the tube containing the preparation from healthy oats at a location corresponding to the depth of the "virus zone." Each sample consisted of 1.0 ml withdrawn from the gradient tube. Approximately 0.2 ml of each sample was removed for shipment to Lincoln for electron microscopy, and the remaining portion of each sample was used in Ithaca for infectivity assays by the membrane-feeding method. The 4 virus preparations and the corresponding preparations made from healthy plants were used for 6 rate-zonal centrifugation experiments. In each experiment, the sample removed from the virus zone contained most of the infectivity and most of the characteristic particles (Table 3).

The predominant particle in samples from the virus zone was polyhedral and about 30 mμ in diameter (Fig. 2). This particle was detected in shadowed, positively stained, or negatively stained preparations. It was difficult to discern individual particles in clumps in preparations with standard light shadow from one direction. Individual particles in clumps could be discerned better in the positively stained preparations. Some preparations from healthy plants (particularly experiment 2-B, Table 3) contained a particle of the same diameter as the virus, but these particles were less dense and flatter. The main difference between particles from healthy oats and the virus was the density, a feature also dependent upon photographic techniques. Therefore, it was not always easy to distinguish these particles from virus in positively stained preparations; whenever there was any doubt, the

TABLE 3

RELATION BETWEEN PARTICLES COUNTED IN ELECTRON MICROGRAPHS AND INFECTIVITY OF SAMPLES OF BARLEY YELLOW DWARF VIRUS REMOVED FROM RATE-ZONAL DENSITY-GRADIENT TUBES

Expt. no.	Buffer used to dissolve sucrose[a]	Sample identification and cm below meniscus[b]	Relative number particles per sample[c]	Infectivity of sample[d]
1-A	0.01 *M* phosphate, pH 7.0	A 1.6	30	0/9, 0/9
		B 2.1	594, 74, 15	8/9, 4/9, 0/9
		C 3.1	14	0/9, 0/9
		H 2.1	2	0/9
1-B	0.05 *M* borate, pH 8.0	A 1.2	6	0/9, 0/9
		B 1.7	663, 159, 18	8/9, 5/9, 0/9
		C 2.7	2	0/9, 0/9
		H 1.7	3	0/9
2-A	0.01 *M* phosphate, pH 7.0	A 1.7	18	0/9, 0/9
		B 2.2	123, 30, 6	4/9, 0/7, 0/9
		C 3.2	7	0/8, 0/9
		H 2.2	4	0/9
2-B	0.05 *M* borate, pH 8.0	A 1.3	28	0/9, 0/9
		B 1.8	248, 61, 15	5/9, 0/9, 0/9
		C 2.8	2	0/9, 0/9
		H 1.8	[illegible]7	0/9
3	0.1 *M* phosphate, pH 7.0	A 1.1	2	0/9, 0/9
		B 1.6	500, 180	9/9, 7/9, 7/9, 3/9
		C 2.6	9	1/9, 0/9
		H 1.6	24	0/9
4	0.1 *M* phosphate, pH 7.0	A 1.1	1	2/9, 0/9
		B 1.6	496, 49, 13	9/9, 9/9, 6/9, 3/9
		C 2.6	3	2/9, 0/9
		H 1.6	0.2	0/9

[a] Gradient columns were prepared 48 hours before use with 4, 7, 7, 7 ml of 100, 200, 300, and 400 mg sucrose per milliliter, respectively, dissolved in the buffer indicated.

[b] Samples A, B, and C were from tubes containing the virus preparation; sample H was from tube containing the control preparation made from healthy plants. Centrifugation was for 3 hours at 23,000 rpm at about 9°C in the SW-25 rotor of the Spinco centrifuge. Since the sample was removed at the indicated depth, the liquid occupied the 2 mm above this depth.

[c] Mean number of characteristic particles in five 2 × 2-inch fields at 15,000 ×; first number is mean count on undiluted sample, successive numbers are counts for 5-fold serial dilutions of sample indicated. All particles were counted after positive staining with uranyl acetate except for experiment 4, where they were shadowed from three directions.

[d] Number of plants that became infected over number infested with 10 aphids (*M. avenae*) that had fed through membranes for about 18 hours on sample indicated before start of 5-day inoculation test feeding period on oat seedlings. First fraction is test of 1:5 dilution, successive fractions are tests on serial 5-fold dilutions of sample indicated. None of 36 plants infested with about 360 control aphids became infected.

particles in preparations from healthy plants were counted.

The best technique of those tried for increasing the contrast of the virus was the three-way shadow, which was used in all later experiments. This left a ring around the upstanding virus particles which did not appear around the flatter particles in preparations from healthy plants. Most virus particles disintegrated in all negative staining techniques tried. Some well-preserved virus particles were found, but the method was not suitable for quantitative results.

Only a trace of infectivity was detected in

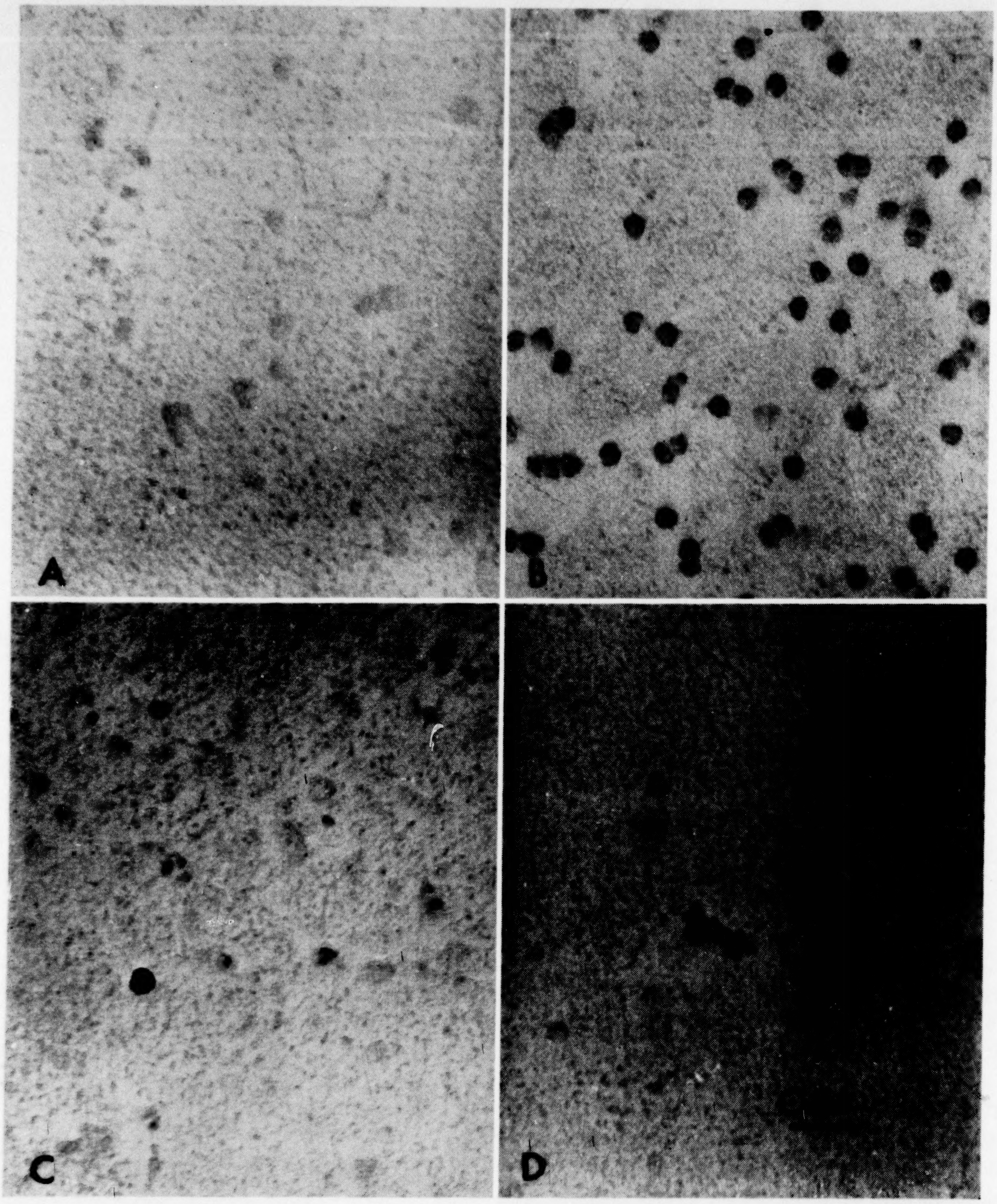

FIG. 2. Electron micrographs showing a portion of typical field of an undiluted sample removed from above the virus zone (A), at the virus zone (B), and below the virus zone (C) from a rate-zonal density gradient tube that was layered with 1.0 ml of a barley yellow dwarf virus preparation concentrated 180-fold. The fourth sample (D) is the control removed from the same depth as the virus zone, but from a tube containing the 180× preparation made from healthy oats. All preparations were shadowed lightly from three directions with tungsten oxide.

2 of the 6 experiments for samples removed from above or below the zone; similarly, very few characteristic particles were observd in these samples (Table 3). No infectivity was detected in any of the samples removed from gradient columns containing the preparation from healthy oats or in any infectivity tests made directly on the original control preparations diluted 1:2. Typical examples of electron micrographs from the 4 samples studied in each experiment are given in Fig. 2 for preparations from experiment 4 (Table 3) in which the original preparations were concentrated 180-fold during 3 cycles of differential centrifugation.

Equilibrium-zonal centrifugation was used

in another experiment involving preparations concentrated 300-fold from either healthy or BYDV-infected oats. One milliliter of each sample was layered on a sucrose column and 1 ml of southern bean mosaic virus was used for the third tube. Following 5 hours at 36,000 rpm, 2 zones were visible in the tube containing BYDV. One zone was 1.2–1.5 cm below the meniscus; the other was at 2.5–2.6 cm. In the tube containing the preparation made from healthy plants, only the upper zone was visible, at 1.1–1.4 cm. Since the upper zone occurred in both tubes, it was not examined further. The tube containing southern bean mosaic virus had a visible zone at 2.4–2.6 cm. Samples (1 ml) were removed for comparison of infectivity and the presence of characteristic particles (experiment 1, Table 4). Only a trace of infectivity was detected in the sample removed from 0.5 cm above the zone and only a few particles were observed in this sample. The sample removed from the zone was highly infectious; it contained the greatest number of characteristic particles. The sample removed from 0.5 cm below the virus zone also was highly infectious and contained a sizable number of particles (experiment 1, Table 4), probably as a result of overlap of the samples. No virus transmission resulted from tests on the control sample removed from the tubes containing the preparation from

TABLE 4

RELATION BETWEEN PARTICLES COUNTED IN ELECTRON MICROGRAPHS AND INFECTIVITY OF SAMPLES OF BARLEY YELLOW DWARF VIRUS REMOVED FROM EQUILIBRIUM-ZONAL DENSITY-GRADIENT TUBES

Expt. no.	Source[a]	Sample identification and cm below meniscus[b]	Relative number of particles per sample[c]	Infectivity of sample[d]
1	Plants	A 2.0	0.03	2/9, 0/9
		B 2.5	27, 18, 2.2, 0.01	9/9, 9/9, 9/9, 6/9
		C 3.0	7.6	9/9, 9/9
		H 2.5	0.16	0/9
2	Aphids	A 1.8	0.07	0/9, 0/9
		B 2.3	1.2, 0.4	3/9, 1/9, 0/9
		C 2.8	8.4	2/9, 0/9
		H 2.3	0.06	0/9

[a] Preparations in experiment 1 were concentrated 300-fold either from healthy or BYDV-infected oats. Preparations in experiment 2 were from 16 g of virus-free or viruliferous aphids (*M. avenae*) adjusted to a final volume of 1.5 ml with 0.1 *M* neutral phosphate buffer.

[b] Gradient columns were prepared 48 hours before use with 0.9 ml each of solutions containing 300, 400, 500, and 600 mg sucrose per milliliter dissolved in 0.1 *M* neutral phosphate buffer. Samples A, B, and C were from tubes containing the virus preparation; sample H was from tube containing the control preparation made from virus-free plants or aphids. Centrifugation was for 5 hours at 36,000 rpm at about 9°C in the SW-39 rotor of the Spinco centrifuge.

[c] Specimens were shadowed from three directions and the particles were counted on the screen of the RCA EMU3G at 105,000 ×. Numbers given are averages per 3 × 4-inch area at this magnification. Particles were counted on at least 25 randomly selected areas when they were numerous. When there were only a few particles per 3 × 4-inch area, the number per 3-inch traverse across a grid opening was counted and divided by 75. At least 7 such traverses were counted for each such sample. Two grids were examined per sample. The first number in each series is for an undiluted sample and subsequent numbers represent successive 5-fold dilutions. The numbers of particles cannot be directly compared with those given in Table 3 because the sizes of areas counted were different.

[d] Number of plants that became infected over number infested with 10 aphids (*M. avenae*) that had fed through membranes for about 18 hours on sample indicated before start of 5-day inoculation test feeding period on oat seedlings. First fraction is test of 1:5 dilution, successive fractions are tests on serial 5-fold dilutions of sample indicated. None of 18 plants infested with about 180 control aphids became infected.

healthy plants or in a direct test on a 1:2 dilution of the control preparation concentrated 300×.

Aphids as source material. Aphids reared on BYDV-infected barley or oat plants were used as source of virus for some experiments. The aphids were removed from source plants by shaking, some were placed directly on oat test seedlings to make certain that they were viruliferous, and the remaining aphids were stored in glass vials at about −20°C. Comparable virus-free *M. avenae* also were frozen. At the start of a purification experiment, partially thawed aphids were ground in buffer in a mortar.

Results of several preliminary experiments suggested that BYDV extracted from aphids had properties similar to virus extracted from plants. For example, in one experiment 16 g of viruliferous *M. avenae* were used as a virus source and 16 g of virus-free aphids were used to make a control preparation (as described below). Since visible zones did not occur in rate-zonal tubes containing the aphid preparations, the southern bean mosaic virus zone was used as the basis for sampling. Reliable particle counts could not be made on the samples because they contained too many impurities. A trace of virus was detected in infectivity assays on the sample removed from above the "virus zone." No virus infectivity was detected in the sample removed from below the "virus zone" or in the sample removed from the "zone" location of the tube containing the preparation made from virus-free aphids. Assay of the sample removed from the "zone" in the tube containing BYDV, however, resulted in infection of 4 of 9 and 3 of 9 plants when dilutions of 1:5 and 1:25 were tested, respectively. None of about 90 aphids tested as controls transmitted virus.

Another preparation was made from 16 g of viruliferous aphids ground at 4° in 30 ml of 0.1 *M* neutral potassium phosphate buffer, 15 ml of chloroform, and 7.5 ml of *n*-amyl alcohol. The preparation was strained through cheesecloth and mixed for 15 minutes at room temperature. Following 15 minutes in an ice bath, the emulsion was broken by centrifugation and the preparation was concentrated by 2 cycles of differential centrifugation. The final volume was adjusted to 1.5 ml with 0.1 *M* neutral phosphate buffer. A parallel preparation was made from 16 g of virus-free *M. avenae*, but most of these aphids had been stored in the freezer for many months longer than the viruliferous aphids. An equilibrium density-gradient centrifugation was carried out with 1.0 ml of each sample and the southern bean mosaic virus zone was used as the basis for removal of samples of 0.5 ml, which were mixed with 0.5 ml phosphate buffer to provide the 1.0 ml needed for assays.

Particle counts again were difficult because of impurities in the samples, particularly in the sample removed from the "virus zone." The sample removed from 0.5 cm below the "virus zone" contained fewer impurities and the largest number of characteristic particles, a result not in serious conflict with results of infectivity tests (experiment 2, Table 4). Taken together, results of tests on preparations made from aphids support the view that BYDV infectivity is associated with the polyhedral particle.

Gel filtration. Gel filtration columns were prepared by suspending G-75 or G-200 Sephadex[4] in neutral phosphate buffer, storing the suspension overnight at 4°, adding additional buffer, and pouring the suspension into columns 2 cm in diameter to allow packing. All experiments were carried out at 21°, and neutral 0.01 *M* or 0.1 *M* phosphate buffer was used as eluant. Successive samples of 5 ml each were collected beginning immediately after 5 ml of the concentrated preparation was placed on the column. The relative concentration of material in each sample was estimated by determining the absorbance of a portion of each sample at 260 and 280 mμ in a Beckman DB spectrophotometer. The remaining portion of each sample was used for infectivity assay at Ithaca and for electron microscope particle counts at Lincoln.

Earlier tests had shown that partially purified preparations were readily separated into two major components by passage through Sephadex and that BYDV infectivity was associated only with the first component eluted from the column. After preliminary experiments had indicated that

the infectious entity and the 30-mμ particle migrated together, a 2 x 16 cm column of G-200 Sephadex was used in another experiment. Five milliliters of a preparation made from infected oats (concentrated 180-fold) was placed on the column and 0.1 *M* neutral phosphate buffer was used as eluant. Forty-six consecutive samples (5 ml each) were collected. Absorbance at 260 mμ and 280 mμ showed the peak for the first fraction to be in sample no. 5; the peak for the second fraction was in sample no. 13. Similar results were obtained with a comparable preparation made from healthy oats. Several of the samples were used to compare the presence of particles with infectivity. Infectivity was associated only with samples from the first peak; these samples also contained the largest number of characteristic particles (Table 5).

TABLE 5

RELATION BETWEEN PARTICLES COUNTED IN ELECTRON MICROGRAPHS AND INFECTIVITY OF SAMPLES OF BARLEY YELLOW DWARF VIRUS COLLECTED FROM SEPHADEX COLUMNS

Sample number and source[a]	Absorbance at 260 mμ	Relative number of particles[b]	Infectivity[c]
2 Virus	0	0.021	0/9, 0/9
3 Virus	0	0.011	1/9, 1/9
4 Virus	0.33	6.0	8/9, 9/9
5 Virus	0.52	6.3	9/9, 8/9
6 Virus	0.19	1.8	8/9, 8/9
7 Virus	0.06	2.3	6/9, 4/9
8 Virus	0.02	0.24	2/9, 1/9
9 Virus	0.01	0.47	1/9, 0/9
13 Virus	0.21	0.005	0/9, 0/9
19 Virus	0	0.014	0/9, 0/9
5 Healthy	0.81	0.007	0/9
13 Healthy	0.16	0.01	0/9
Aphid control	—	—	0/9

[a] Number of 5 ml sample collected consecutively from column of G-200 Sephadex[4] (2 × 16 cm) using 0.1 *M* neutral phosphate buffer as eluant. Samples were collected immediately after 5 ml of preparation concentrated 180-fold either from BYDV-infected oats or from healthy oats was placed on top of column.

[b] Specimens were shadowed from three directions and particles were counted on the screen of the RCA EMU3G at 105,000 ×. Numbers given are averages of 3 × 4-inch area at 105,000 ×. Particles were counted on at least 34 areas selected at random. If the average number of particles was less than one per area, particles were counted on ten 3-inch traverses of grid openings and the numbers were divided by 75. Two grids were examined per sample.

[c] Number of plants that became infected over number infested with 10 aphids (*M. avenae*) that had fed through membranes for about 18 hours on sample indicated before start of 5-day inoculation test feeding period on oat seedlings. First fraction is test of 1:2 dilution, second fraction is test of 1:10 dilution.

DISCUSSION

Plant viruses can be divided into two main categories as follows: those that are mechanically transmissible, such as tobacco mosaic virus and southern bean mosaic virus; and those that are not generally mechanically transmissible, such as aster yellows virus and BYDV. Most of the plant viruses that have been purified are of the mechanically transmissible type. Little is known about viruses that are not mechanically transmissible, most of which are transmitted by leafhoppers, because they are generally difficult to purify (Black, 1955). Leafhopper-transmitted viruses that have been purified have proved to be much larger and often of more complicated morphology than the viruses which are readily transmitted mechanically (see Maramorosch, 1963).

BYDV is an example of the smaller group of viruses transmitted by aphids in a circulative or persistent manner and not mechanically transmissible to plants. In contrast to the leafhopper-transmitted viruses, the BYDV particle is similar in size to many polyhedral viruses of the mechanically transmissible group. For example, BYDV and southern bean mosaic virus sediment at almost the same rate and are indistinguishable in the electron microscope. It will remain for future work to determine the physical and chemical characteristics of BYDV that are responsible for its unique physiological relationships with both plants and animals.

A major limiting factor in this work was the low virus content of infected plants. The concentration of BYDV in plants probably is restricted by its tissue specificity and

necrosis of phloem tissue that limits synthesis of the virus. Use of better methods of extraction and purification and use of other source plants may make possible greater recovery of virus, but low virus concentration in infected plants will no doubt continue to be a major limitation in studies on BYDV. Aphids might be a good source of virus, but rearing large numbers of viruliferous aphids usually presents practical problems. Moreover, aphids appear to contain a high concentration of materials that make virus purification difficult.

The low concentration of BYDV in plants in itself may have some relevance to host-virus relations. This low concentration could account for the fact that BYDV is not mechanically transmissible to plants, but it is not necessarily the most important factor. Despite the low concentration of virus in plants infected by BYDV, the disease is as severe as that of other plants infected by viruses that accumulate in high concentration, another illustration of the fact that disease severity and virus concentration are not necessarily related. The low concentration of virus, the tissue specificity, and the type of symptoms all suggest that the physiological basis of disease is quite different for barley yellow dwarf than for mosaic diseases.

Although results reported here are for a strain of BYDV transmitted specifically by *M. avenae*, a similar particle has been found in tests on another vector-specific strain of the virus that is transmitted by *Rhopalosiphum padi* (L.), and not by *M. avenae*. These tests on the second strain of BYDV were based on rate- and equilibrium-zonal centrifugation of 4 separate virus preparations. Further comparisons among different strains of BYDV are in progress.

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Radiosensitivity of Bacteriophage P22

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Radiosensitivity of *Salmonella* phage P22 and factors modifying radiosensitivity were investigated.

When P22 phage were irradiated by X-rays in nonprotective medium, marked resistance was exhibited by about 10^{-5} of the population. A mutant isolated from the resistant population showed resistance mainly to the indirect effects and the aftereffects of X-rays. The resistance seemed to be caused by some changes in the protein part of the phage particle that is responsible for the adsorption of the phage to bacterial cells.

A 1:100 dilution of X-irradiated phage suspension into nonirradiated physiological saline prevented further inactivation, a result suggesting that phage had no remaining potential damages. When phage were irradiated in nonprotective medium and kept in the same medium, aftereffects were observed for at least 96 hours.

The processes of adsorption and injection of P22 phage were almost completely resistant to ultraviolet light and to direct effects of ionizing radiations. On the other hand, indirect effects of X-rays greatly affected adsorption, but to a lesser extent injection. Heat treatment at 80° without irradiation strongly affected both adsorption and injection, presumably because of denaturation of phage protein. When phage were irradiated indirectly by X-rays, the frequency of transduction of histidine loci of *Salmonella typhimurium* by the phage decreased in proportion to the decrease of surviving phage, possibly owing to the failure of adsorption and injection.

DNA is the major target of inactivation by ultraviolet light and by direct effects of ionizing radiations. In contrast, inactivation of the tail protein of the phage particles appears to be important for indirect effects of ionizing radiation.

INTRODUCTION

The radiosensitivity of bacteriophage to ionizing radiation is determined by the DNA contents of phage particles when the inactivation is restricted to the direct effects of ionizing radiations (Epstein, 1953; Garen and Zinder, 1955; Bertani, 1960; Terzi, 1961). Exceptions are the phages that contain single-stranded DNA (Tessman *et al.*, 1957). Bacteriophage irradiated with X-rays in a solution of inorganic salts are inactivated more than in protective media, such as nutrient broth (Luria and Exner, 1941; Watson, 1950, 1952). The protective media are supposed to protect against the effects of free radicals and peroxides produced in the solution by ionizing radiation, thereby eliminating indirect effects and aftereffects and yielding direct effects only.

Direct effects and aftereffects of X-rays on transductions by *Salmonella* phage P22 were studied by Takebe and Hartman (1962). The results indicated that the radiosensitivity of the genetic material of P22 bacteriophage and of the transductional segment of bacterial chromosomes carried by P22 phage were proportional to their respective DNA contents.

The inactivation of bacteriophage is generally detected as inactivation of plaque formation, involving either of three steps: adsorption of phage by host bacterial cells, DNA injection by adsorbed phage, and new

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Interaction and Mutual Suppression among Three Strains of Aster Yellows Virus

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Three isolates of aster yellows virus, collected in California, were differentiated on the basis of symptoms on common plantain, *Plantago major* L., *Nicotiana rustica* L. var. *humilis*, aster,[1] and periwinkle and were designated as the Severe, Dwarf, and Tulelake strains. Leafhopper transmission tests conducted on 20 species of plants indicated only minor host range differences between the three strains. Cross protection experiments on plantain and *N. rustica* provided evidence that plants infected with either Dwarf or Severe strains were protected from infection by Tulelake strain. Protection was reciprocal between the Dwarf and Severe strains. In contrast, in plantain plants infected with Tulelake strain and challenged by the Dwarf or Severe strain the original strain was replaced and symptoms of the challenging strain developed. The most unexpected results were obtained when *N. rustica* plants infected with Tulelake strain were challenged with either Dwarf or Severe strain. This antagonistic interaction resulted in mutual suppression of both the Tulelake and challenging strain in each combination and the development of symptomless plants. Leafhoppers acquired virus from 14 of 23 such plants. The Tulelake strain was recovered from 7 plants, and both the Tulelake strain and the challenging strain were acquired from the seven other plants.

INTRODUCTION

This investigation was undertaken to describe, differentiate, and determine the interaction of three strains of the aster yellows virus.

Kunkel (1926) first described the aster yellows virus from the eastern United States and demonstrated that the aster leafhopper *Macrosteles fascifrons* (Stål) was the vector. A comparison of the host ranges of the two virus strains indicated that certain hosts such as celery and zinnia were readily susceptible to the western isolate but not to the eastern one (Kunkel, 1932; Severin, 1934). The strains were not distinguished on the basis of symptoms.

Kunkel (1955) demonstrated in cross protection tests that the aster yellows virus from the eastern United States was related to a western strain from southern Oregon. Gladiolus plants grown in Florida from infected Oregon corms were the source of the western strain. The 2 strains were differentiated on the basis of symptoms which developed on aster, periwinkle, zinnia, and *N. rustica*.

Freitag (1958, 1963) reported the occurrence of three strains of aster yellows virus in California and briefly summarized results of cross protection tests.

Frazier and Posnette (1958) described aster yellows virus as being lethal to strawberry. The virus from strawberry developed different symptoms on plantain, aster, carrot, *Daucus carota* L. var. *sativa* DC., and celery than did the stock greenhouse culture. Virus transfers made to plantain and aster with leafhoppers fed on naturally infected celery, filaree, *Erodium moschata* L'Her., horseweed, *Erigeron canadensis* L., and Indian tobacco, *Nicotiana bigelovii* Wats., also

[1] Scientific names for plant hosts not given in text are presented in Table 1.

produced variant symptoms and suggested the occurrence of different virus strains in California.

Kunkel (1955), Maramorosch (1958), and Freitag (1958, 1963) reported plant virus strain interactions in their leafhopper vectors.

Valenta (1959b, c) carried out cross protection tests with several yellows-type plant viruses. He found cross protection to occur between certain yellows viruses, but in other combinations tested, he found that the challenge inoculations resulted either in dual infections or more often in suppression of one virus by another. He suggested that a "gradient of mutual interaction" existed between the viruses. In some instances he found that plants originally infected with one strain became temporarily symptomless carriers of the viruses.

The interaction of plant viruses resulting in either synergistic or antagonistic effects has been widely established. In the synergistic interaction, plants simultaneously infected with two viruses develop a disease with more severe symptoms than those produced by either of the viruses alone. The opposite antagonistic reaction has been commonly used to demonstrate the close relation of virus strains. In this type of interaction, a plant infected with one strain of a virus is protected from infection by a closely related strain, but not by an unrelated virus.

Bawden and Kassanis (1945) indicated a third type of interaction in which a second virus such as severe etch can suppress and replace a virus such as potato virus Y, or Hyoscyamus virus 3, which is already established in a plant.

A fourth type of interaction is described in the present paper; it involves the interaction between certain strains of the aster yellows virus. When a plant infected with one strain is infected with a second strain, there results an antagonistic effect that produces a mutual suppression of both strains in the same plant host.

MATERIALS AND METHODS

Infective aster leafhoppers, *Macrosteles fascifrons* (Stål), were reared on common plantain, *Plantago major* L., infected with one of the strains of aster yellows virus (Severe, Dwarf, or Tulelake). Noninfective leafhoppers reared on Sacramento barley, *Hordeum vulgare* L., plants were tested at various times to ensure their freedom from virus. Stock leafhopper colonies were maintained in the greenhouse in cylindrical cages consisting of plywood rings with 4 wooden posts covered with lawn cloth on 3 sides and the top. The cages had a glass front through which observations could be made. They were 8 inches in diameter and varied in height from 10 to 16 inches. Cages of the same type were used to confine leafhoppers on plants during the inoculation experiments. All plants were grown from seeds. The plantain plants were usually inoculated by leafhoppers after development of from 4 to 6 leaves whereas the *N. rustica* plants were inoculated when they were about 3 inches tall and in the 4- to 8-leaf stage.

ORIGIN AND SYMPTOMS OF STRAINS

Severe. The strain of aster yellows virus designated as the Severe strain is considered to be the most prevalent one in California and is the virus described originally by Severin (1929) from celery, lettuce, *Lactuca sativa* L., and other plants. This strain has been maintained in the greenhouse at Berkeley for more than thirty years. However, no careful record has been kept of the original source, and additional field collections of the virus have been added to the stock greenhouse culture at intervals. The Severe strain is characterized on plantain by vein-clearing, chlorosis, elongation, twisting, and intertwining of petioles (Fig. 1A). The flowers usually develop prominent phyllody and virescence. The Severe strain is best differentiated from the other strains by the spindly growth, severe chlorosis, etiolation, elongation, and proliferation (Fig. 1B and 3A) produced on *Nicotiana rustica* L. var. *humilis*. The symptoms on aster (Fig. 2A) and periwinkle (Fig. 2B) are somewhat less distinctive, but can serve in some instances to differentiate the strains.

Dwarf. Evidence that more than one strain of aster yellows virus occurred in California was first noted in 1956 when individual leaf-

FIG. 1. A. Common plantain, *Plantago major* L., infected by 3 strains of aster yellows virus; left, Severe strain; center, Dwarf strain; and right, Tulelake strain. (× 1/3) B. Terminal shoots of *Nicotiana rustica* L. var. *humilis*: left, healthy; and infected by: left center, Tulelake strain, showing severely dwarfed leaves with swollen wavy veins; right center, Dwarf strain, with small green leaves, phyllody and virescence; right, Severe strain, with abnormal flowers, chlorosis, and elongation of shoots. (× 2/3)

FIG. 2. Terminal shoots from plants infected by 3 strains of aster yellows virus. A. Aster, left, Severe strain, showing chlorosis, proliferation, virescence, and phyllody; center, Dwarf strain with virescence and phyllody; right, Tulelake strain showing chlorosis and floral rosette. B. Periwinkle, left, Tulelake strain; center, Dwarf; right, Severe; all showing virescence and phyllody. Terminal shoots infected by Severe or Tulelake strains are chlorotic whereas the shoot infected by the Dwarf strain remained green. (× 2/3)

hoppers, during latent period tests, transmitted virus which resulted in different symptoms developing on separate plants of common plantain. Noninfective leafhoppers fed on plants showing these variant symptoms would then transmit to other test plants only virus that caused similar symptoms. The Dwarf strain isolate was readily differentiated from the greenhouse stock culture of the Severe strain. Its field origin is obscure, although another Dwarf strain isolate was recovered from Ladino clover, *Trifolium repens* L., collected near Galt, California, in November 1960. A third Dwarf strain isolate was obtained from cosmos, *Cosmos bipinnatus* Cav., naturally

infected with aster yellows and collected in Berkeley during November 1963. Plantain infected with Dwarf strain shows initial symptoms of mild vein-clearing, curvature of petioles, and intertwining. The plants gradually lose these early symptoms, become severely stunted and develop dark green, dwarfed, strap-shaped leaves (Fig. 1A). The Dwarf strain can be readily distinguished on *Nicotiana rustica* L. var. *humilis* by the stunting and the development of numerous small shoots which bear dwarfed green leaves (Fig. 1B) and which produce a bunchy top effect (Fig. 3B and 3F). The pronounced phyllody and virescence and lack of chlorosis on aster (Fig. 2A) and on periwinkle (Fig. 2B) makes it easy to distinguish the Dwarf strain from the Severe and the Tulelake strains.

Tulelake. The third strain was originally obtained from potato, *Solanum tuberosum* L., naturally infected with aster yellows virus at Tulelake in the northeastern section of California. The symptoms of this strain on plantain are similar to those of the Severe strain and it is difficult to differentiate them (Fig. 1A). The Tulelake strain, however, produces a somewhat more pronounced vein clearing and chlorosis. An additional symptom which sometimes occurs on plantain is a dark brown exudate on the petioles of infected plants. The most striking symptoms are produced on *Nicotiana rustica* L. var. *humilis* where severe stunting (Fig. 3C and 4F), chlorosis, prominent bright yellow venation and leaf rolling are characteristic. The terminal leaves (Fig. 1B) are severely dwarfed and thickened and develop swollen, wavy, irregularly shaped veins. Symptoms which develop on aster (Fig. 2A) and periwinkle (Fig. 2B) are less readily differentiated from those of Severe strain. There is a tendency for Tulelake strain to produce more stunting and to inhibit the development of flowers on some hosts such as aster.

RESULTS

Host Range Studies of the Three Strains

Twenty species of plants were inoculated by leafhoppers to determine whether the susceptibility of certain plant hosts could be used as a basis for differentiating the 3 strains. The plants were inoculated by feeding 20 viruliferous leafhoppers on each plant for 1 week. From 5 to 12 plants of each species being tested were inoculated with each of the 3 strains of the virus. Infections were determined only by the development of symptoms (Table 1) and indicated that 4 species of plants, sugar beet, soybean, globe amaranth, and Turkish tobacco, failed to develop infection with any strain. Other species, such as *Nicotiana glutinosa* L., Marglobe tomato, white sweet clover, and zinnia, showed some resistance to all 3 strains, and usually only 50% or less of the plants inoculated developed infection. Inoculation of other species resulted in 50% or more of the plants developing infection with all 3 strains. Among these, *Nicotiana rustica* L. var. *humilis*, China aster, alsike clover, and periwinkle, proved to be the most susceptible species to all 3 strains. Although common plantain was not included in this test, previous work and the results given in Table 2 provide ample evidence for considering this species the most susceptible one to all 3 strains. The results in Table 2 show that 164 of 170 plantain plants tested were infected when 2–4 leafhoppers were fed on each plant. Although berseem clover and Apple-of-Peru (Table 1) were readily infected with Severe strain, they failed to develop infection with Dwarf strain, but these tests are somewhat too limited to be sure of any real differences in this respect. Except for these results, there was no evidence that the 3 strains could be readily separated on the basis of the susceptibility of any of the species tested. The symptoms produced by the 3 strains on certain host plants such as *N. rustica*, aster, and periwinkle provided the best means for distinguishing them. Kunkel (1955) was the first to utilize these three hosts as indicator plants for differentiating strains of aster yellows virus.

Interactions in Plantain

Cross protection tests were conducted with the 3 virus strains in common plantain. In one type of interaction studied, the first virus strain was inoculated by leafhoppers feeding on test plants for 1 week; this was followed during the next week by the challenging

TABLE 1

LEAFHOPPER TRANSMISSION OF THREE STRAINS OF ASTER YELLOWS VIRUS TO 20 SPECIES OF PLANTS

Common name	Scientific name	No. plants infected/no. plants inoculated		
		Severe	Dwarf	Tulelake
Aster	*Callistephus chinensis* Nees	12/12	11/12	10/12
Celery	*Apium graveolens* L. var. *dulce* Pers.	8/12	6/12	10/12
Alsike clover	*Trifolium hybridum* L.	5/5	5/5	5/5
Berseem clover	*T. alexandrinum* L.	5/5	0/5	2/5
Red clover	*T. pratense* L.	8/11	10/11	7/11
Crimson clover	*T. incarnatum* L.	4/6	5/6	3/6
Hubam clover	*Melilotus alba* Desr. var. *annua* Coe	5/5	2/5	2/5
White sweet clover	*M. alba* Desr.	3/5	2/5	2/5
Birdsfoot trefoil	*Lotus corniculatus* L.	6/10	5/10	5/10
Soybean	*Glycine max* Merr.	0/6	0/6	0/6
Globe amaranth	*Gomphrena globosa* L.	0/6	0/6	0/6
Sugar beet	*Beta vulgaris* L.	0/5	0/5	0/5
	Nicotiana glutinosa	2/6	1/6	3/6
Rustic tobacco	*N. rustica* L. var. *humilis*	12/12	12/12	12/12
Turkish tobacco	*N. tabacum* L.	0/6	0/6	0/6
Marglobe tomato	*Lycopersicon esculentum* Mill.	0/6	1/6	3/6
Apple-of-Peru	*Nicandra physalodes* Gaertn.	5/5	0/5	0/5
Zinnia	*Zinnia elegans* Jacq.	3/11	1/11	4/11
Periwinkle	*Vinca rosea* L.	4/6	6/6	6/6
White Bush scallop squash	*Cucurbita pepo* L.	5/5	5/5	4/5

TABLE 2

CROSS PROTECTION TESTS WITH THREE STRAINS OF THE ASTER YELLOWS VIRUS IN *Plantago major* L.[a]

Strain inoculated during first week	Challenging strain inoculated during second week	Number of plants infected/inoculated	Resulting symptoms on *Plantago major*
Dwarf	—	20/20	20 Dwarf
Dwarf + Severe	—	20/20	12 Severe, 6 Dwarf, 2 Intermediate
Dwarf	Severe	20/20	11 Dwarf, 2 Severe, 7 Intermediate
Dwarf	Tulelake	10/10	10 Dwarf
Severe	—	15/20	15 Severe
Severe + Tulelake	—	10/10	6 Severe, 4 Tulelake?
Severe	Tulelake	10/10	7 Severe, 3 Tulelake?
Severe	Dwarf	19/20	18 Severe, 1 Dwarf
Tulelake	—	10/10	10 Tulelake
Tulelake + Dwarf	—	10/10	9 Dwarf, 1 Tulelake
Tulelake	Dwarf	10/10	10 Dwarf
Tulelake	Severe	10/10	8 Severe, 2 Tulelake?

[a] Each strain was inoculated to a plant by means of two leafhoppers.

virus. In other tests, the 2 strains were inoculated simultaneously, and as a check on the method and on the infectivity of the leafhoppers a series of plants was inoculated with only a single strain. The results (Table 2) indicated that plantain was readily infected with any of the 3 strains and that a high percentage (75–100%) of the plants

developed infection. A plant was inoculated by allowing 2 leafhoppers transmitting the strain to feed on it for a week.

When common plantain was inoculated by Dwarf the first week and then challenged by Severe during the second week, the plants developed mainly symptoms of Dwarf and most were protected from Severe. All 20 plants tested showed Dwarf symptoms at first (Table 2), but later 7 of these developed intermediate symptoms, and 2 ultimately showed symptoms of the challenging Severe strain. The Dwarf virus provided only partial protection against the Severe strain in plantain. When the sequence of the strains was reversed (Severe first, followed by Dwarf) 18 of the 20 plants tested developed symptoms of Severe only, 1 plant showed dwarf symptoms, and another failed to develop any symptoms. The results indicated that Severe provided good protection against Dwarf in plantain.

When plants were inoculated simultaneously by leafhoppers with Severe and Dwarf strains, the Severe strain appeared to dominate because 12 of 20 plants developed Severe symptoms while 6 of 20 showed Dwarf symptoms and 2 exhibited some symptoms of both strains. Common plantain inoculated with Dwarf and Severe either simultaneously or first with one strain and then with the other usually developed symptoms of only one strain. However, some plants would first show symptoms of one and then later also symptoms of the second strain.

The Tulelake strain failed to protect against Dwarf when common plantain was inoculated the first week with Tulelake and challenged during a second week by Dwarf. All plants developed symptoms of Dwarf. When plants were inoculated with Tulelake and Dwarf strains simultaneously, Dwarf again predominated in nearly all plants tested. It was difficult to assess the results of tests conducted to determine the interaction of Tulelake and Severe strains because of the problem of differentiating the symptoms of these two strains on common plantain. However, the best evaluation of the results as shown in Table 2 indicate that both Severe strain and Dwarf usually predominated over Tulelake strain.

Noninfective leafhoppers were fed on plants inoculated with two strains, some showing only symptoms of one strain, and others showing symptoms of both strains, to determine what strains were present and could be acquired by leafhoppers feeding on such plants. The insects usually recovered the virus which had induced symptoms. From 14 plants inoculated with both Dwarf and Severe, but showing Dwarf, the leafhoppers acquired and transmitted only Dwarf to 64 of 101 test plants. From 10 plants inoculated with both Severe and Dwarf, but showing Severe, the insects transmitted only Severe to 44 of 68 test plants. Both strains of the virus were acquired by leafhoppers from 8 plants which showed some symptoms of each and resulted in 28 plants developing Dwarf symptoms and 11 Severe symptoms on the 61 plants used in the test. The leafhoppers in one instance recovered only Severe strain from plantain showing Dwarf symptoms and in other instances acquired both strains from plants showing only symptoms of one strain.

Interactions in Nicotiana rustica L. var. humilis

Nicotiana rustica L. var. *humilis* plants were inoculated with various combinations of strains by feeding leafhoppers, transmitting a desired strain, on a plant for a week. When one strain had been inoculated, this inoculation was followed a week later by inoculation with a second strain. Control plants were inoculated at the same time with a single strain during the first week. The data presented in Table 3 give the results of virus strain interactions in *N. rustica* plants. In the first test carried out in January, 5 plants were inoculated at the same time with all combinations indicated in Table 3, both single and dual strain inoculations. In the second and third trials made during March and April the single strain inoculations were compared to those in which the Tulelake strain was challenged by either Severe or Dwarf strains. During May, the 3 tests consisted of comparing various combinations of dual strain inoculations.

The strain that was introduced first nearly always predominated and prevented the development of all but slight symptoms of

the second strain. The inoculation of *N. rustica* plants with Dwarf strain the first week and Severe the second (Table 3) resulted in 29 of 30 plants developing symptoms of Dwarf (Fig. 3E) with a slight chlorosis indicating the presence of the Severe strain. In another sequence when 30 plants were first inoculated with Severe strain and then a week later by Dwarf strain, 24 developed Severe symptoms (Fig. 4A), 4 Dwarf and 1 intermediate. In this instance, plants exhibiting mainly symptoms of the Severe strain also showed evidences of being dually infected by being less chlorotic than plants known to be infected by the Severe strain alone (Fig. 4B).

When *N. rustica* plants were inoculated simultaneously with the Dwarf and Severe strains, one or the other strain usually predominated. Of 25 plants so inoculated symptoms of Severe strain developed on 12 and of Dwarf (Fig. 4C) on 7 (Table 3). A slight modification of symptoms indicated the influence of the second strain.

When 10 leafhoppers viruliferous with Dwarf and 10 viruliferous with Tulelake were fed simultaneously for 1 week on *N. rustica* plants, the Dwarf strain predominated (Table 3) with a mild chlorosis and slight delay in the development of symptoms suggesting the presence of the Tulelake strain. These results might indicate a more rapid multiplication or the preempting of multiplication sites by the Dwarf strain. However, observations made on the development of symptoms of the Tulelake and Dwarf strains in *N. rustica* as in other host plants failed to indicate that the Dwarf strain develops symptoms more rapidly than the Tulelake strain. The reverse is usually the case—plants infected with Tulelake strain showing definite symptoms sooner than plants infected with Dwarf strain.

The Dwarf strain dominated when *N. rustica* plants were inoculated during the first week with it, and then challenged by Tulelake during the second week (Fig. 3D). The results indicated a cross protection reaction with a suppression of the development of the symptoms of the challenging strain. Inocula-

TABLE 3

Cross Protection Tests with Three Strains of the Aster Yellows Virus in *Nicotiana rustica* L. var. *humilis*: Each Plant Inoculated by Ten Leafhoppers for Each Strain[a]

Strain inoculated during first week	Challenging strain inoculated during second week	Number of plants infected/inoculated	Symptoms on *Nicotiana rustica*
Dwarf	—	14/15	14 Dwarf
Dwarf + Severe	—	19/25	7 Dwarf (some severe effect, plants more severely dwarfed and with slight chlorosis), 12 Severe
Dwarf + Tulelake	—	5/5	5 Dwarf (delayed symptom development and slight chlorosis)
Dwarf	Tulelake	5/5	4 Dwarf (slight chlorosis) 1 Tulelake
Severe	—	11/15	11 Severe
Severe + Tulelake	—	5/5	5 Tulelake only (leafhoppers apparently failed to transmit Severe)
Severe	Tulelake	5/5	4 Tulelake only, 1 Severe (poor transmission of severe strain suspected)
Dwarf	Severe	29/30	29 Dwarf (slight chlorosis indicating some severe effect)
Tulelake	—	20/20	20 Tulelake
Tulelake	Severe	45/45	28 Recovered, 17 partially recovered
Tulelake	Dwarf	45/45	39 Recovered; 5 partial recovery, 1 Tulelake
Severe	Dwarf	29/30	24 Severe, 4 Dwarf, 1 Intermediate

[a] Tests were conducted in January, March, April, and May of one year.

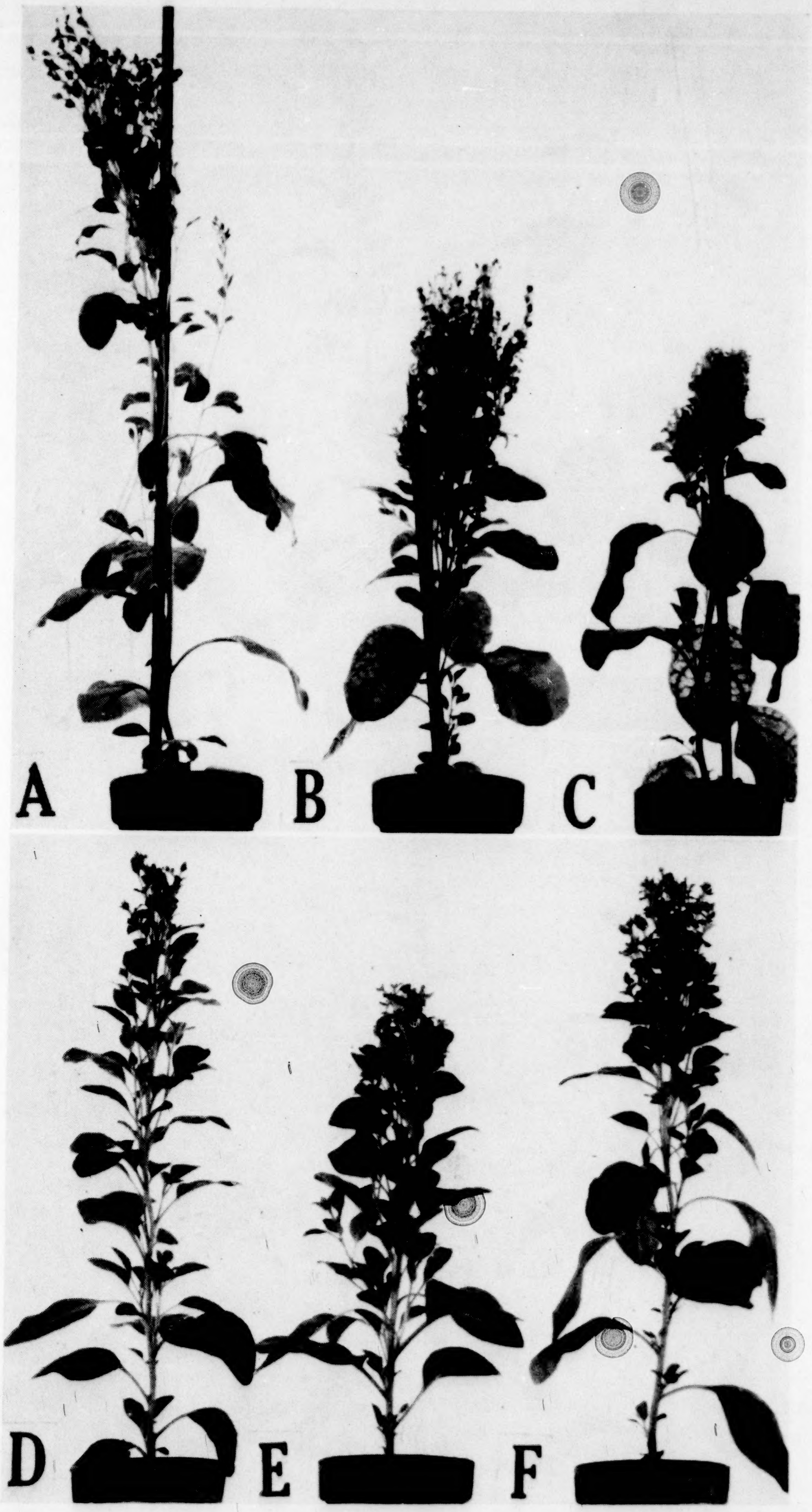

FIG. 3. *Nicotiana rustica* L. var. *humilis* experimentally infected with 3 strains of aster yellows virus. A. Severe strain producing elongation. B. Dwarf strain producing numerous dwarfed green leaves. C. Tulelake strain producing numerous dwarfed chlorotic terminal shoots. D. Inoculated first with Dwarf strain then challenged with Tulelake strain, plant shows Dwarf symptoms. E. Inoculated with Dwarf then challenged with Severe strain, plant shows Dwarf symptoms. F. Inoculated with Dwarf strain only. (× 1/7)

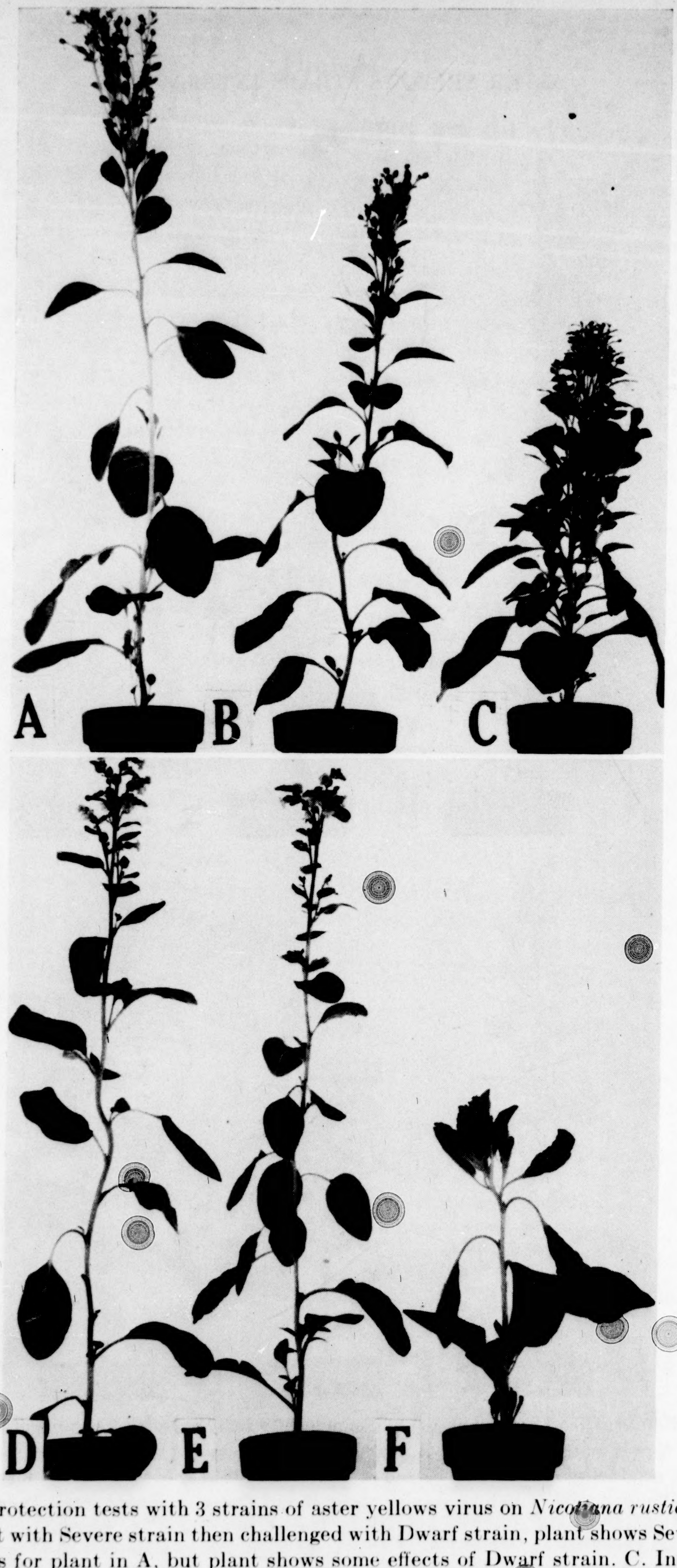

FIG. 4. Cross protection tests with 3 strains of aster yellows virus on *Nicotiana rustica* var. *humilis*. A. Inoculated first with Severe strain then challenged with Dwarf strain, plant shows Severe symptoms. B. Inoculations as for plant in A, but plant shows some effects of Dwarf strain. C. Inoculated simultaneously with Severe and Dwarf strains, but plant shows Dwarf symptoms only. D. Inoculated with Tulelake strain, then challenged with Dwarf strain, plant shows normal growth and flowers. E. Inoculated with Tulelake strain then challenged by Severe, plant shows normal flowers and growth. F. Inoculated only with Tulelake strain and showing typical symptoms of stunted, rolled leaves with swollen veins. (× ⅐)

TABLE 4

RESULTS OF TESTS WHEN *Nicotiana rustica* var. *humilis* WAS FIRST INOCULATED BY TULELAKE STRAIN AND THEN CHALLENGED AT VARIOUS INTERVALS BY EITHER DWARF OR SEVERE STRAIN[a]

Challenging strain	Number of plants of 15 inoculated showing	Interval between inoculation with Tulelake strain by 10 leafhoppers and inoculation with challenging strain					Subtotals[b]
		3 Days	7 Days	10 Days	14 Days	21 Days	
Severe	Recovery	5	3	9	11	9	37
	Severe	4	4	1	3	0	12
	Tulelake	4	8	5	1	5	23
	No symptoms	2	0	0	0	1	3
Dwarf	Recovery	6	11	7	9	6	39
	Dwarf	1	1	0	1	0	3
	Tulelake	8	3	7	5	8	31
	No symptoms	0	0	1	0	1	2

[a] *N. rustica* control plants inoculated with a single strain at the same time, resulted in 10 infected of 10 inoculated with Tulelake strain, 10 infected of 15 inoculated with Severe strain, and 11 infected of 15 inoculated with Dwarf strain. These experiments were conducted during late fall, winter, and spring. None of these control plants recovered from symptoms.

[b] Subtotals represent the numbers of plants exhibiting each of the four types of response which followed inoculation of 75 plants with a challenging strain.

tion first with Severe and then Tulelake or simultaneously by these 2 strains resulted in the development of Tulelake symptoms in 9 of 10 plants (Table 3). In view of the more extensive tests described below where plants were first inoculated by Tulelake and then challenged by Severe, the results of this test were considered to be due to inefficient transmission of the Severe strain in these tests rather than to cross protection by the Tulelake strain.

The most provocative results were obtained when *N. rustica* plants were first inoculated with Tulelake strain and then inoculated a week later by either the Dwarf strain or the Severe strain. In both instances the plants first developed a definite and pronounced vein-yellowing, indicating infection by the Tulelake strain. This was followed by what was judged to be a complete recovery in 67 of 90 plants and partial recovery in 22 of 90 plants as shown in Table 3. Only one of 90 plants developed the typical Tulelake syndrome. In the recovered plants the new growth was normal with few if any detectable symptoms that could be attributed to either the Tulelake strain or the challenging strain. The flowers and leaves were normal in

TABLE 5

LEAFHOPPER ACQUISITION OF STRAINS OF ASTER YELLOWS VIRUS FROM PLANTS, INFECTED WITH TWO STRAINS WHICH HAD SHOWN RECOVERY FROM SYMPTOMS

Nicotiana rustica Inoculated with	Number of plants tested	Number of plants from which leafhoppers acquired:			
		Tulelake	Severe or Dwarf	Both strains	Neither strain
Tulelake and Severe	12	4	0	2	6
Tulelake and Dwarf	11	3	0	5	3

appearance and suggested that an antagonism between the two strains resulted in a mutual suppression of both strains so that the new growth developed into apparently normal, healthy plants (Fig. 4D and E). In most instances the older leaves that had developed the initial vein yellowing symptoms persisted for a long time on the recovered plants. The interaction of the strains in these instances suggests a phenomenon of mutual suppression which results in neu-

tralization of the two competing strains by one another.

Kunkel (1941) demonstrated inactivation of aster yellows virus and Valenta (1959a) of yellows type viruses when infected plants were kept at 40°C from 4 to 21 days. During the present experiments the greenhouse temperatures generally varied from 16° to 27°C and rarely exceeded this. They were never high enough for a sufficiently long period to inactivate the aster yellows virus in *N. rustica*. No recovery from symptoms was observed in any of the 45 control plants inoculated at the same time with only one of the three strains, in the experiments carried out in January, March, and April. Similarly, none of the 65 plants inoculated by leafhoppers during the month of May with various combinations of Severe and Dwarf showed any signs of recovery from conspicuous or well-pronounced symptoms. In contrast *N. rustica* plants inoculated at the same time with Tulelake strain and challenged by Severe or Dwarf showed a definite partial to complete recovery from symptoms.

Nicotiana rustica var. *humilis* plants which had been inoculated with Tulelake strain by exposing them to the feeding of viruliferous leafhoppers for 3 days, were then inoculated with either Severe or Dwarf strain by feeding leafhoppers on them for 3 or 4 days after an interval of 3, 7, 10, 14, or 21 days had elapsed. The results of these tests (Table 4) indicate that recovery from symptoms by plants previously inoculated with Tulelake strain may follow if the plants are also inoculated with a second strain from 3 to 21 days after inoculation by the first strain. The length of the elapsed interval between inoculation of the first strain and the challenging strain did not appear to affect the number of plants which recovered from symptoms. Control plants inoculated with a single strain at the same time and kept in the greenhouse under identical conditions showed no signs of recovery. Since these tests were done during late fall, winter, and spring of 1958–1959, possible inactivation of the virus by high temperatures in the greenhouse was very unlikely.

Experiments were conducted to ascertain whether leafhoppers could acquire either one or both strains from these dually infected plants which had recovered from symptoms. Fifty noninfective leafhoppers were fed for about 7 days on each of 23 recovered plants (Table 5) and then transferred to healthy plantain and *N. rustica* plants at intervals of a week or more. The insects acquired the Tulelake strain from 7 of these plants and both strains (either Dwarf or Severe together with Tulelake) from 7 others. No transmission resulted from the remaining 9 of the 23 plants, and in no instance did the leafhoppers acquire either Dwarf or Severe strain alone from these dually infected plants, all of which showed few if any symptoms of either of the two virus strains with which they had been inoculated. Failure of leafhoppers to acquire virus from 9 of the 23 plants favors the hypothesis of mutual suppression of the two interacting strains and suggests complete cure. However, noninfective leafhoppers, fed on plants showing symptoms of the initial strain inoculated and which failed to develop symptoms of the challenging strain, readily acquired the initial strain (9 of 10), and acquired both the initial and the challenging strain in only 1 of 10 trials.

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In Vitro and *in Vivo* Effects of Commercial Tannic Acid and Geranium Tannin on Tobacco Mosaic Virus[1, 2]

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A water extract of common geranium (*Pelargonium hortorum* Bailey) leaves inhibited development of starch lesions in cucumber cotyledons when applied by vacuum infiltration 24 hours after inoculation with tobacco mosaic virus (TMV). The active principle in the geranium extract was related to tannic acid. Tannic acid, had an LD_{50} of 1.4×10^{-4} *M*. Complete inhibition of starch lesions was obtained by infiltrating an effective dosage at any time between 1 hour and 33 hours after virus inoculation; between 33 and 72 hours, inhibition decreased progressively. Infiltration at 24 hours after inoculation of the systemic host *Physalis floridana* resulted in reduced virus titer.

Tannic acid infiltration reduced the total host ribonucleic acid (RNA) in cucumber cotyledons during the first few hours after infiltration, but the cotyledons recovered within 24 hours. Virus titer assays on treated cucumber cotyledons revealed a linear increase in virus replication to higher levels than in untreated leaves, even though the number of starch lesions was reduced. In the cucumber-TMV system, tannic acid prevented the development of starch lesions and interfered with the host defense mechanism, thus permitting systemic virus invasion.

Tannic acid inactivated both TMV and TMV RNA *in vitro* by forming noninfectious complexes. TMV RNA was much more sensitive to inactivation than was whole TMV.

TMV appeared to have three *in vitro* reactions with tannic acid: an instantaneous reaction that was reversible with caffeine: a slow first-order reaction partially reversible with caffeine: and a denaturing reaction, at high TMV concentrations, not reversible with caffeine. The tannic acid reaction with TMV RNA was caffeine reversible. Part of the inactivation of whole TMV appeared to be due to a reaction of tannic acid with the RNA core.

A brown coloration resulted when tannic acid was allowed to react with TMV, TMV RNA, uridylic acid, or guanylic acid. This reaction did not occur with TMV protein, other nucleotides, nucleosides, or purine or pyrimidine bases. The reaction was thus specific for uridylic and guanylic acids.

INTRODUCTION

Numerous plant extracts have been investigated for plant virus-inhibiting agents (Manil, 1949; Bawden, 1954; Yoshii *et al.*, 1955). In general, virus inhibitors thus obtained are presumed to be of two types. They either form complexes with the virus particles rendering them noninfective, or they affect the inoculation sites of the host plants, preventing virus particles from establishing infection. These can be classified as exogenous inhibitors which interfere with or prevent the normal entrance of virus particles. Other phases of the virus infection process, such as the release of virus nucleic acid and the paths of biochemical synthesis of virus nucleic acid and protein within the

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cell, are possibilities for inhibitory action. Reduction of virus titer and production of noninfectious virus particles containing a foreign purine base resulting from treatment with 8-azaguanine (Matthews, 1954; Lindner *et al.*, 1960) well illustrate the other possibilities for virus inhibition. The modification of tobacco mosaic virus (TMV) by passage through certain hosts (Lindner *et al.*, 1961), and the fact that many plant species show physiological resistance against virus infection, would indicate the possible influence on virus synthesis of specific agents existing in the plant cell.

In searching for inhibiting agents from various plant extracts (including mushrooms, lichens, mosses, ferns, and flowering plants), we found that geranium (*Pelargonium hortorum* Bailey) extract is effective in preventing starch lesion formation in cucumber cotyledons caused by TMV infection. This paper deals with the studies of geranium extract and its related effective component, tannic acid, on TMV-infected plants and on TMV and its nucleic acid (TMV RNA).

MATERIALS AND METHODS

Kirkpatrick and Lindner (1954) described a vacuum infiltration method of applying chemotherapeutic compounds to plants previously inoculated with virus. Frozen geranium leaves were homogenized with three volumes of laboratory distilled water in a Waring blendor. The homogenate, passed through one layer of cheesecloth, was heated to 60° for 20–30 minutes, then centrifuged at low speed (10,000 *g* for 10 minutes) and at high speed (112,000 *g* for 1 hour). The extract was then neutralized by the addition of 1 *M* KOH. Further dilutions of this extract were made for vacuum infiltration of cucumber seedlings inoculated 20–24 hours previously with purified TMV. In general, unless otherwise mentioned, 14 cotyledons were infiltrated as replicates for each treatment.

For biological assay, Chicago pickling cucumber (*Cucumis sativus* L.) seedlings were grown in 2½-inch pots in the greenhouse. Ten to 15 days after seeding, they were in the proper stage for virus inoculation. The cotyledons were inoculated by the airbrush method (Lindner and Kirkpatrick, 1959) with purified TMV (0.05 μg/ml) in distilled water or purified TMV RNA (0.1 μg/ml) in 0.02 *M* neutral phosphate buffer containing 2% by weight of 600-mesh Carborundum. Starch lesions were counted 6–7 days after inoculation. Inoculated leaves were harvested and stored 24 hours in the dark in a moist chamber to remove excess starch. The chlorophyll was removed by heating with 70% ethanol in an 80° water bath. Starch lesions were developed by placing the cotyledons in an IKI–lactic acid (1:20) mixture.

TMV was prepared from infected leaves of *Physalis floridana* Rydb. by homogenizing leaves with 3 volumes by weight of 0.01 *M* neutral phosphate buffer containing 0.01 *M* cysteine HCl. The homogenate was filtered through cheesecloth and clarified at 60° for 30 minutes; the TMV was purified by repeated differential centrifugation. A stock solution, in distilled water at 4°, was used in the work reported here. Concentrations of TMV were calculated from absorption measurements on a Beckman model DU spectrophotometer. A similar procedure was also used for virus titer assay of different samples. TMV RNA was isolated from the virus by the phenol method of Gierer and Schramm (1956) carried out in a 4° room. The virus was first suspended in 0.02 *M* phosphate buffer at pH 7.3, and extraction was carried out in that medium. After the first alcohol precipitation, the TMV RNA pellet was redissolved with 0.02 *M* acetate buffer at pH 5.5, and the alcohol precipitations were repeated twice. The final TMV RNA in acetate buffer was then frozen and stored at −20° until used.

All *in vitro* reactions of TMV and TMV RNA with tannic acid were carried out in 0.02 *M* acetate buffer at pH 5.5. The tannic acid used was obtained from Matheson, Coleman, and Bell and had a reported molecular weight of 1701.18.

RESULTS

A. *Effects of Geranium Extract and Tannic Acid on TMV in Vivo*

The active component in geranium extract. Aqueous geranium extracts inhibited TMV starch lesion formation in cucumber cotyle-

dons. In general, a 1:2 dilution of the original extract, when vacuum infiltrated to cucumber seedlings 24 hours after virus inoculation, inhibited development of 99% of the starch lesions. At a dilution of 1:4 there was 93% inhibition; at 1:7, 65% and at 1:10, 20% inhibition. However, cotyledons infiltrated with the 1:2 dilution suffered marginal burn and later appeared chlorotic. The 1:4 dilution of geranium leaf extract produced only slight yellowing. The active principle inhibiting the starch lesion development was heat stable (autoclaved at 15 pounds for 20 minutes), dialyzable, and soluble in ethanol. The extract precipitated egg albumin and gelatin, gave positive tests for glucose, and a blue color with ferric chloride.

Paper chromatography of both alcohol and water extracts of geranium leaves (butanol–acetic acid–water as solvent) identified two compounds, gallic acid and a component similar to tannic acid, by their respective R_f values and ultraviolet absorption spectra. Gallic acid on paper chromatograms was most prominent when heat-clarified leaf extract was used. Fresh leaf juice applied directly to the paper produced only a faint gallic acid spot. Tannic acid formed a streak, rather than a spot, on paper chromatograms in the many solvents tried.

The tannic acid of commerce (Chinese gallotannin) is reported in the "Biochemists' Handbook" (1961) to be a "glucose skeleton acylated with residues of gallic acid, *m*-digallic acid and, possibly, trigallic acids." The tannin from geranium leaves is probably also a mixture. While no molecular weight can be assigned to such a mixture, for comparative purposes it was convenient to use the 1701 molecular weight for tannic acid given by the Merck Index. This same weight, for convenience, was used for geranium tannin.

Geranium leaves contain relatively large amounts of tannin. Assays based on a standard ultraviolet absorption curve of commercial tannic acid indicated an average of 2% tannin in fresh geranium leaves. Freshly expressed geranium leaf juice, after high speed centrifugation, gave an ultraviolet absorption spectrum similar to that of commercial tannic acid (Fig. 1).

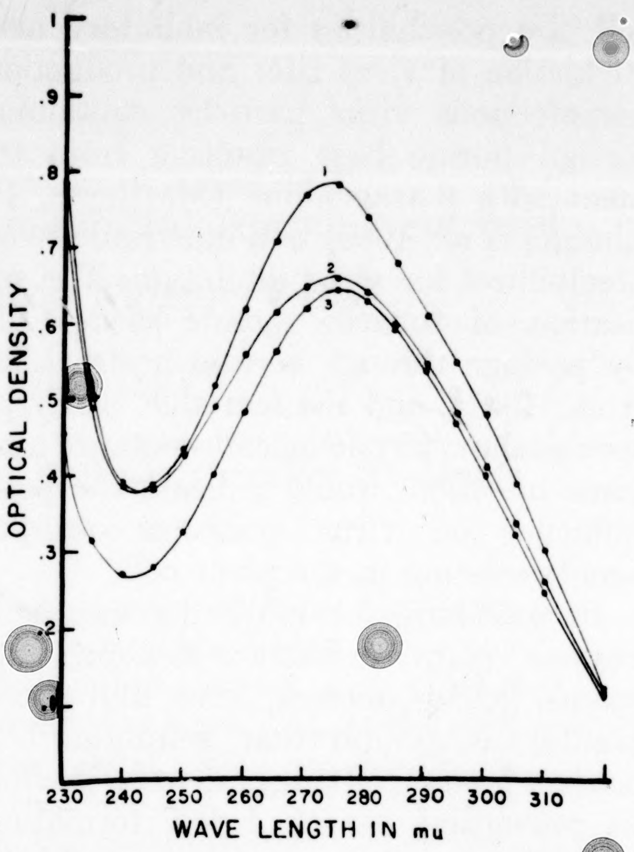

FIG. 1. Ultraviolet-absorption spectra of (*1*) geranium leaf extract (1:1500 dilution), (*2*) active TMV starch lesion inhibiting component isolated from geranium leaves on paper chromatograms, and (*3*) commercial tannic acid (15 μg/ml).

In cucumber cotyledons, infiltration of 3.5×10^{-4} *M* tannic acid completely inhibited development of the starch lesions, even at 33 hours after virus inoculation. Complete inhibition was obtained at any time between 1 hour and 33 hours after virus inoculation. Infiltration of the tannic acid (3.5×10^{-4} *M*) 48 hours after inoculation resulted in an 83% inhibition of starch lesions, and infiltration 56 hours after inoculation inhibited 42% of the lesions. Insignificant inhibition of starch lesion development occurred when tannic acid was infiltrated 72 hours after inoculation.

Effect of tannic acid on TMV replication in cucumber plants. Starch lesion formation is a host response to virus infection. Factors preventing the formation of starch lesions incited by the virus do not necessarily inhibit virus development. Therefore, the effect of tannic acid is a direct inhibitory effect against the formation of starch lesions, a side effect resulting from virus infection. It is not necessarily a direct measurement of the inhibiting effect on the establishment of virus infection sites by tannic acid.

Cucumber cotyledon samples infiltrated with different concentrations of tannic acid and distilled water 24 hours after TMV inoculation were harvested and assayed at different time intervals for their total virus titer. Separate samples (25 cotyledons) were then weighed, frozen, homogenized, and heat clarified; the TMV in each sample was purified by differential centrifugation. The final clear virus solutions from different cotyledon samples were assayed by spectrophotometric readings at 260 mμ and by infectivity comparisons with dilutions of each sample. Samples of cotyledons were also collected and cleared; the number of starch lesions were recorded to indicate the extent of inhibition by treatment. The biological infectivity assay agreed with the spectrophotometric readings that the active virus concentration was high in samples infiltrated with higher concentrations of tannic acid. The results of 1 of 5 experiments are shown in Fig. 2. At the high concentration of tannic acid (3×10^{-4} *M*) 91% of the infection sites produced no starch lesions. At 2×10^{-4} *M*, 64% of the infection sites produced no lesions and at 1×10^{-4} *M*, 15% of the infection sites were affected. Infiltration with low concentrations of tannic acid resulted in no increase in virus titer beyond 7 days after inoculation (Fig. 2). The two highest concentrations of tannic acid, however, resulted in linear increase of virus titer up to 21 days after inoculation, indicating further virus replication beyond the limitation of starch lesions.

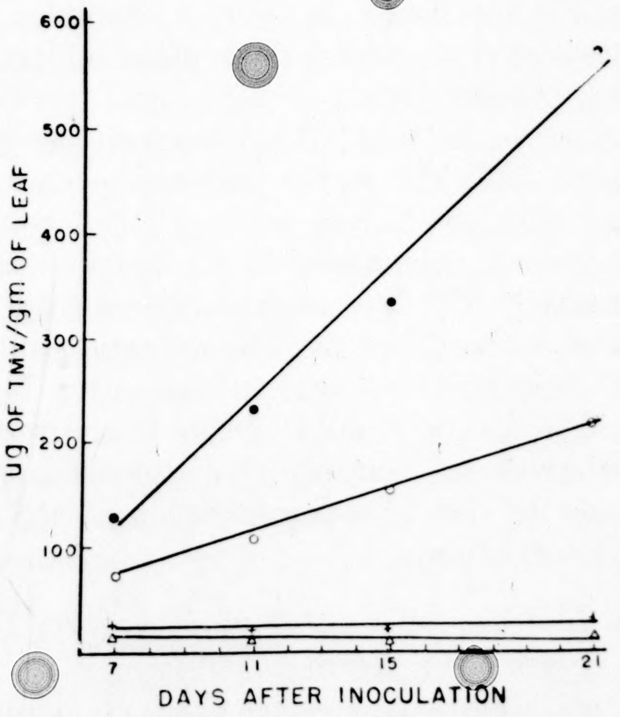

FIG. 2. Virus titers at various times after inoculation of infected cucumber cotyledons infiltrated with different concentrations of tannic acid 24 hours after inoculation. ●——●, 3×10^{-4} *M* tannic acid; ○——○, 2×10^{-4} *M* tannic acid; +——+, 1×10^{-4} *M* tannic acid; △——△, distilled water.

Infiltration of tannic acid in previous experiments was carried out 24 hours after virus inoculation. In order to determine the effect of time of treatment, cucumber plants inoculated with TMV were infiltrated at different intervals after inoculation. Distilled water-infiltrated plants served as controls. On the 11th day after inoculation, samples were harvested, weighed, and assayed for total virus titer and total number of starch lesions (Table 1). Infiltration of tannic acid up to 36 hours after inoculation increased the virus titer. The 24-hour treatment was the most effective with a 27-fold increase. There were progressively more starch lesions as the interval between inoculation and treatment was increased.

Systemic spread of TMV in cucumber induced by tannic acid treatment. Cotyledons of 30 cucumber plants were inoculated with TMV. The primary leaves were shielded by tinfoil to prevent accidental infection. Twenty-four hours later, 20 plants were infiltrated (cotyledons and primary leaves)

TABLE 1

THE EFFECT OF TANNIC ACID (2×10^{-4} *M*) INFILTRATED AT DIFFERENT INTERVALS AFTER TMV INOCULATION (0.25 μG/ML) ON THE PRODUCTION OF STARCH LESIONS AND THE TOTAL VIRUS TITER OF CUCUMBER COTYLEDONS (20 FOR EACH SAMPLE), ASSAYED 11 DAYS AFTER INOCULATION

Infiltration time (hours after virus inoculation)	Tannic acid		Distilled water	
	Virus titer (μg/g of leaf)	Average number of starch lesions	Virus titer (μg/g of leaf)	Average number of starch lesions
0	510	0	13	53
3	609	2	22	206
6	752	12	29	251
24	917	19	34	257
36	351	175	80	284
48	111	229	42	248

with 3×10^{-4} *M* tannic acid and the remaining 10 plants with distilled water to serve as a control. During the first 2 weeks, noninoculated primary leaves of 10 of the tannic-acid-infiltrated cucumber plants were further treated daily by brushing with 10^{-3} *M* tannic acid plus 0.1% aerosol O.T. After 40 days, expanded terminal leaves from all three sets of cucumber plants were collected and weighed. They were then homogenized separately in three volumes of 0.02 *M* neutral phosphate buffer in a Virtis homogenizer. Further dilution to 1:10 with buffer was made for infectivity assay on cucumber cotyledons. An average of 62 lesions per cotyledon was obtained in the assays of the samples that had been infiltrated and brushed with tannic acid, 3 lesions per cotyledon in the assays of the samples infiltrated with tannic acid only, and 0 lesion per cotyledon in samples infiltrated with distilled water.

Accidental contamination of virus during daily brushing was unlikely because both tannic acid and aerosol O.T. inactivate TMV *in vitro*. Owing to the hairy and wrinkled texture of primary leaves, vacuum infiltration does not produce a complete water-soaked condition in primary leaves as compared with cotyledons. Thus infiltration alone was less effective than daily brushing.

Effect of tannic acid on TMV replication in a systemic host. P. floridana plants trimmed to two leaves were inoculated with purified TMV 24 hours before vacuum infiltration with 2×10^{-4} *M* tannic acid or with distilled water. After 1- and 2-week intervals, inoculated leaves and young growing leaves were harvested separately, weighed, and kept frozen for virus titer assay. One week after infiltration with tannic acid (based on one typical experiment out of three), the inoculated leaves contained 0.65 mg of virus per gram (fresh weight) of leaves while the control inoculated leaves (infiltrated with distilled water) contained 2.5 mg/g. After 2 weeks, the difference in virus titer was not significant: 6.3 mg/g for the treated leaves and 6.6 mg/g for the control leaves. In systemically infected new growth, the results were 0.38 mg of virus per gram for the treated leaves and 0.78 mg/g for the control leaves for the 1-week samples and the 2-week samples, 7.4 mg/g for the tannic acid treatment, and 11.1 mg/g for the control. Therefore, infiltration with 2×10^{-4} *M* tannic acid reduced virus titer about 75% during the first week after inoculation. Infiltration with tannic acid 6 days after TMV inoculation had no effect on virus titer as compared with the water-infiltrated check. Virus purified from treated plants showed the same specific infectivity as that from the untreated plants.

The effect of tannic acid on RNA synthesis in cucumber cotyledons. Since tannic acid had a marked effect in increasing TMV replication in cucumber and a depressing effect on TMV replication in *P. floridana*, it was of interest to determine the effect of tannic acid on normal RNA synthesis in cucumber. Four groups of cucumber seedlings were infiltrated with tannic acid at concentrations of 0.0, 1.9, 3.8, and 5.7×10^{-4} *M*. At 1, 2, and 4 hours after infiltration, samples were taken from each group, weighed, and frozen. The total RNA content of each cotyledon sample was determined according to the method of Ogur and Rosen (1950). The total RNA after extraction was measured by ultraviolet absorption at 260 mμ. These results are shown in Fig. 3. Samples collected at 1, 3, and 9 days after infiltration with tannic acid showed no significant reduction in total RNA content as compared with the water infiltration controls. The effect of tannic acid on RNA content of the cell thus occurred at the time of infiltration. The host cells recovered from this effect within 24 hours. The reduction in total RNA content at early hours after tannic acid infiltration could be due to a direct interference of normal RNA synthesis or it could be due to a precipitation of RNA in the cotyledon.

B. *In Vitro Effects of Tannic Acid on TMV and TMV RNA*

The effect of tannic acid on TMV. Addition of tannic acid to TMV causes an increase in viscosity of the solution, and further increases in tannic acid concentration produces a gel-like formation when the TMV concentration is above 3 mg/ml. TMV protein isolated by the glacial acetic acid method of

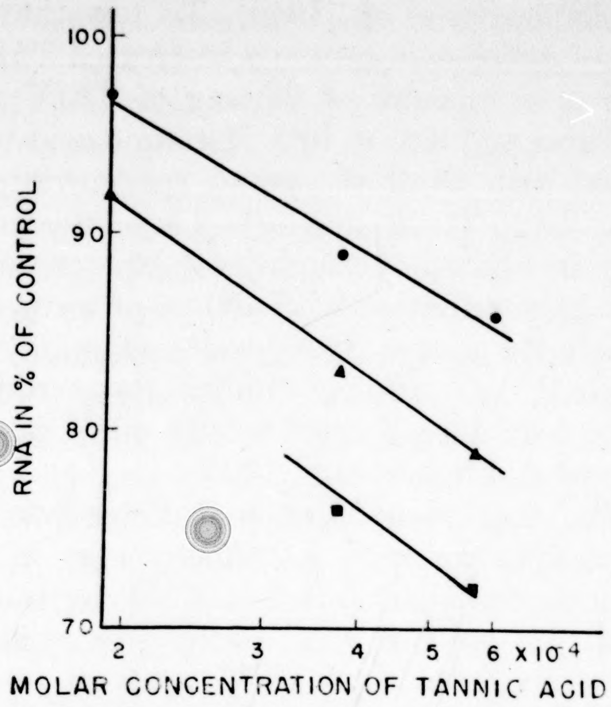

FIG. 3. The reduction in total RNA content in cucumber cotyledons vacuum-infiltrated with different concentrations of tannic acid. ■——■, Samples collected 1 hour after infiltration; ▲—▲, samples collected 2 hours after infiltration; ●——●, samples collected 4 hours after infiltration.

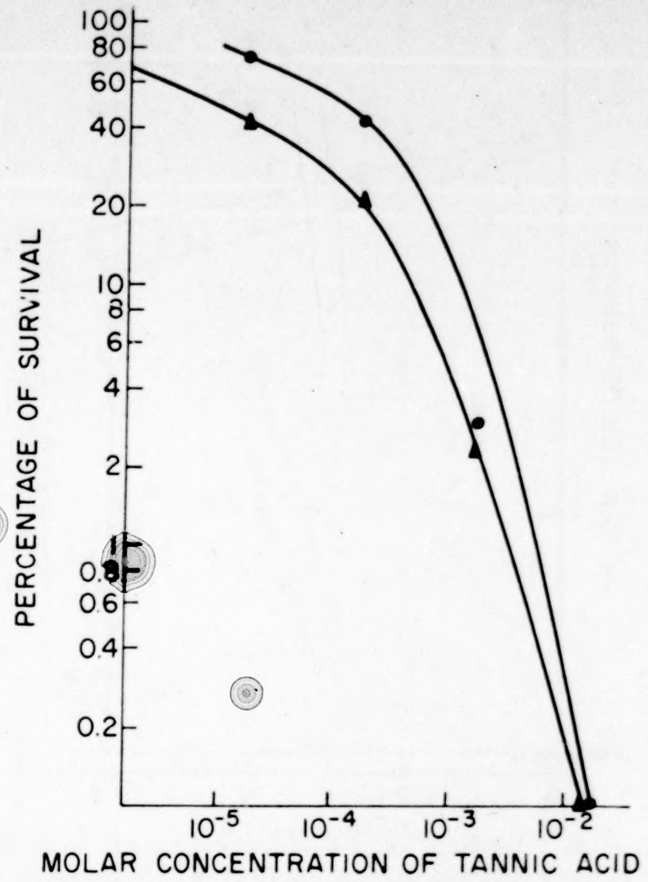

FIG. 4. The effect of tannic acid concentration on TMV inactivation when incubated at 35°C for 4 hours (●——●) and for 24 hours (▲——▲). The virus concentration was 5.5 mg/ml.

Fraenkel-Conrat (1957) exhibited a similar increase in viscosity with addition of tannic acid. The inactivation curves of 5.5 mg/ml TMV after treatment at 35° with different concentrations of tannic acid for 4 hours and 24 hours are shown in Fig. 4. The tannic acid treated virus was diluted 1–50,000 with distilled water for infectivity assay. The sharp downward drop of both curves at high tannic acid concentrations (2×10^{-3} and 2×10^{-2} M) is coincident to the gel-like formation of the reaction mixture at these concentrations. In similar experiments using a lower concentration of TMV (1 mg/ml) there was no visible increase in viscosity nor gel-like formation at the highest concentration of tannic acid tried. The inactivation curve then maintained the original initial slope (Fig. 7).

In a time-interval study, the inactivation of TMV by tannic acid seemed to be of two different types. The first reaction was instantaneous, and the second reaction seemed to follow first order kinetics (Fig. 5).

TMV at different concentrations (0.1, 1, 10, 100, and 1000 μg/ml) was treated with a constant molar ratio of tannic acid at 35° for 4 hours and then diluted with distilled water to 0.1 μg/ml of virus for infectivity assay. If dilution alone will restore infectivity of tannic acid-treated TMV, the percentage of infectivity should be the same for all treatments. However, this was not the case, as indicated in Table 2. The percentage of infectivity decreased as the concentration of tannic acid increased in the reaction mixture.

Centrifugation studies also indicated that dilution in distilled water would not dissociate the tannic acid-TMV complex. The tannic acid-treated virus was highly diluted with distilled water and centrifuged (112,000 g); the pellet was rinsed and resuspended in distilled water for infectivity assay. There was no increase in infectivity.

However, suspension of tannic acid-treated virus in tris(hydroxymethyl)aminomethane (Tris) buffer at pH 9.0 partially restored infectivity. In neutral phosphate buffer (0.02 M) the treated virus (2.25 mg/ml TMV in 3.8×10^{-3} M tannic acid for 4 hours at 25°) had 21% of the original infectivity, while in Tris buffer (0.02 M) at pH 9.0 the same treated virus had 61% of the original infectivity. Similarly, tannic

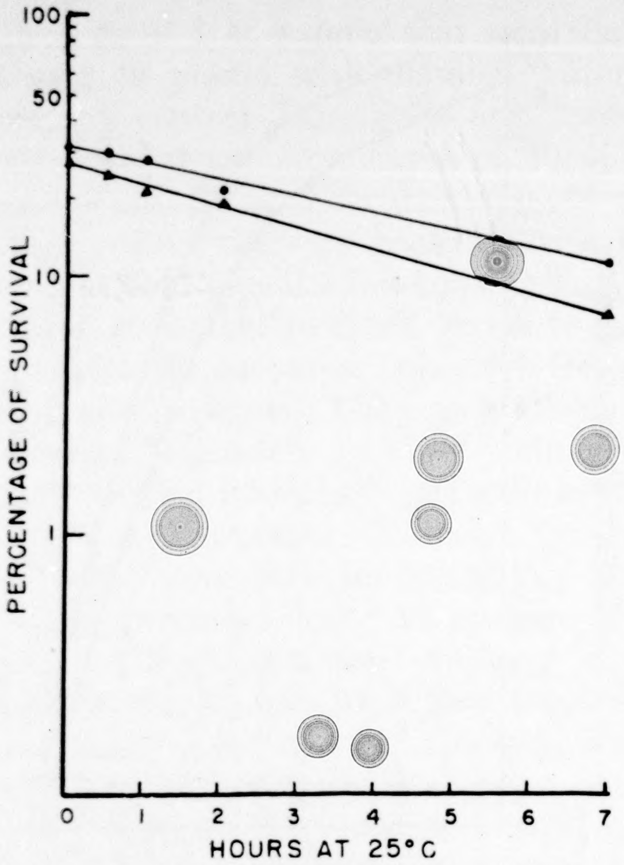

FIG. 5. Rate of inactivation of TMV (3.0 mg/ml) by tannic acid when incubated at 25°C for different time intervals. ●——●, TMV + 2 × 10^{-4} *M* tannic acid; ▲——▲, TMV + 2 × 10^{-3} *M* tannic acid.

TABLE 2

THE EFFECT OF DILUTION ON TANNIC ACID-TREATED TMV[a]

Concentration of TMV (μg/ml)	Concentration of tannic acid	Dilution for inoculation	Percentage of infectivity[b]
0.1	10^{-7} *M*	1:1	79
1	10^{-6} *M*	1:10	34
10	10^{-5} *M*	1:100	19
100	10^{-4} *M*	1:1000	13
1000	10^{-3} *M*	1:10000	3

[a] Reaction mixtures at constant molar ratio between TMV and tannic acid were incubated in a water bath at 35°C for 4 hours. They were further diluted to 0.1 μg of TMV per milliliter with distilled water for infectivity assay. For each sample 14 cotyledons were inoculated.

[b] Percentage of lesion formation as compared with nontreated TMV inoculated at 0.1 μg/ml.

acid-treated virus, when tested in acetate buffer (0.02 *M*) at pH 5.0 had an infectivity level of 40%.

Caffeine has been used successfully to regenerate protein from an insoluble protein–tannic acid complex (Mejbaum-Katzenellenbogen *et al.*, 1959). To test the effect of caffeine on tannic acid-treated TMV, 5 μl of a mixture of 2.5 mg of TMV per milliliter and 3.8 × 10^{-3} *M* tannic acid was mixed with 50 μl of various concentrations of caffeine (1–10 mg/ml). These mixtures were incubated overnight at room temperature before dilution with 100 ml of water for infectivity assay. The results (Fig. 6) indicated that caffeine, under these conditions, could regenerate about 40% of the tannic acid-inactivated TMV.

The time of addition of caffeine was an important factor in its effectiveness in inhibiting the inactivation of TMV by tannic acid. If caffeine was added immediately after tannic acid and TMV had been mixed, and the resulting mixture was immediately diluted and assayed, there was essentially no effect on TMV infectivity. On the other hand, if TMV was incubated with tannic acid for 7 hours and then treated with caffeine, 76% of the original infectivity was recovered. Similarly, a 24-hour incubation period before adding caffeine resulted in a 46% recovery. If tannic acid and TMV were allowed to form a gel at high concentrations,

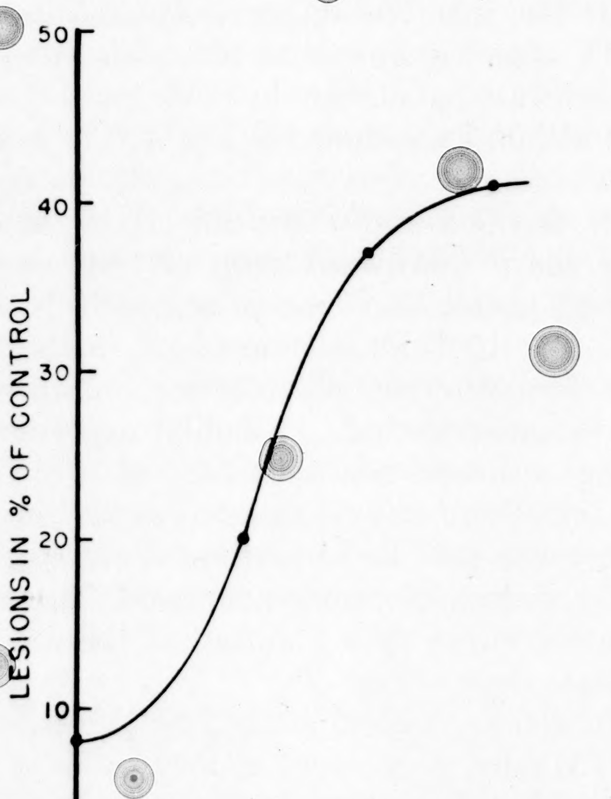

FIG. 6. The effect of various concentrations of caffeine on the regeneration of tannic acid-treated TMV.

caffeine treatment at room temperature did not bring TMV into solution, and no infectivity was recovered.

Comparative effects of tannic acid on TMV and TMV RNA. The effect of tannic acid on TMV and isolated TMV RNA *in vitro* was determined by treating TMV and TMV RNA at room temperature with different concentrations of tannic acid for 2 hours in 0.02 *M* acetate buffer at pH 5.5. All treatments were diluted and inoculated for infectivity assay. Since the particle weight of TMV is 20 times higher than that of TMV RNA, the concentration of TMV in reaction with tannic acid was 1 mg/ml and that of TMV RNA was 50 μg/ml. In a separate experiment, a concentration of 1 mg of TMV RNA per milliliter was used for comparison. After 2 hours' reaction at room temperature, the samples were diluted to 0.1 μg/ml in case of TMV RNA and 0.05 μg/ml in case of TMV for infectivity assay. The inactivation curves of TMV and TMV RNA show different slopes (Fig. 7), indicating that TMV RNA is more readily inactivated than whole TMV.

The effect of the period of incubation of TMV RNA with tannic acid at two different temperatures is presented in Fig. 8. Autoclaved geranium extract centrifuged at 112,000 *g* was calculated to contain the equivalent of 2×10^{-4} *M* of tannic acid through spectrophotometric readings at 273 mμ. The inactivation curve of RNA by this autoclaved geranium extract is also presented in Fig. 8. Components other than tannin in the geranium extract may also function as inactivating agents to TMV RNA. Autoclaving the extract should eliminate the action of ribonuclease. However, the similarity of the inactivation curves for tannic acid and geranium tannin would indicate that geranium tannin is the principal inhibitor in the extract.

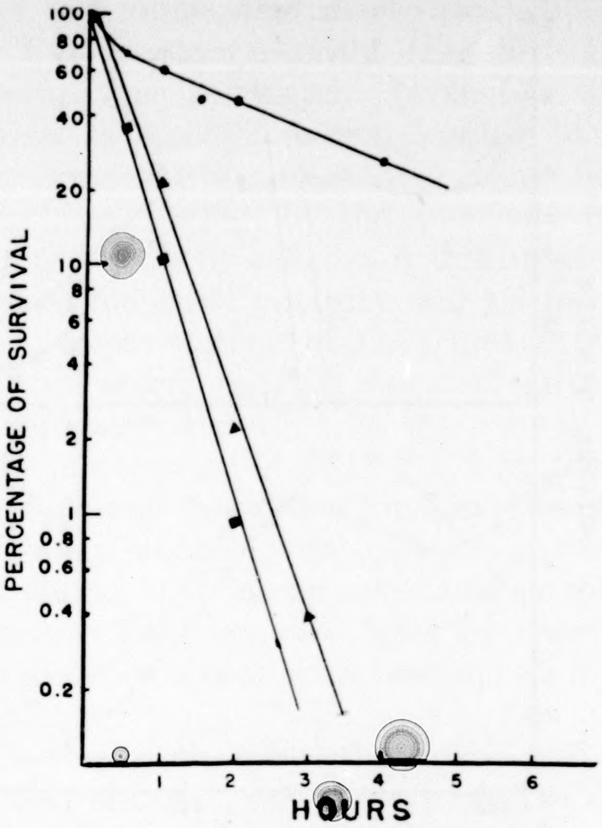

FIG. 8. Inactivation of TMV RNA (1 mg/ml) by 10^{-4} *M* tannic acid (●——●) at 25°C and 2×10^{-4} *M* tannic acid (▲——▲) at 35°C, and by autoclaved geranium extract (■——■) containing the equivalent of 2×10^{-4} *M* of tannic acid at 35°C.

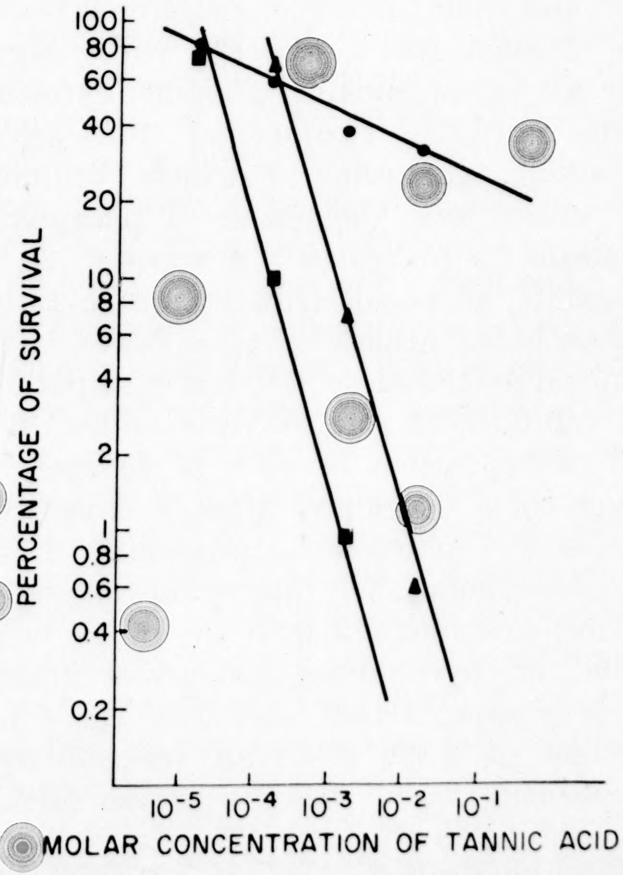

FIG. 7. The inactivation of TMV at 1 mg/ml (●——●) and TMV RNA at 50 μg/ml (■——■) and 1 mg/ml (▲——▲) by different concentrations of tannic acid for 2 hours at room temperature.

Regeneration of tannic acid-inactivated TMV RNA by caffeine was also tried. Recovery of infectivity was obtained by treating the noninfective particles with caffeine solution at a concentration range of 2.5–20 mg/ml in an ice bath for several hours or for 1 hour at room temperature. Complete recovery of original infectivity was obtained.

Spectral shift of TMV and TMV RNA

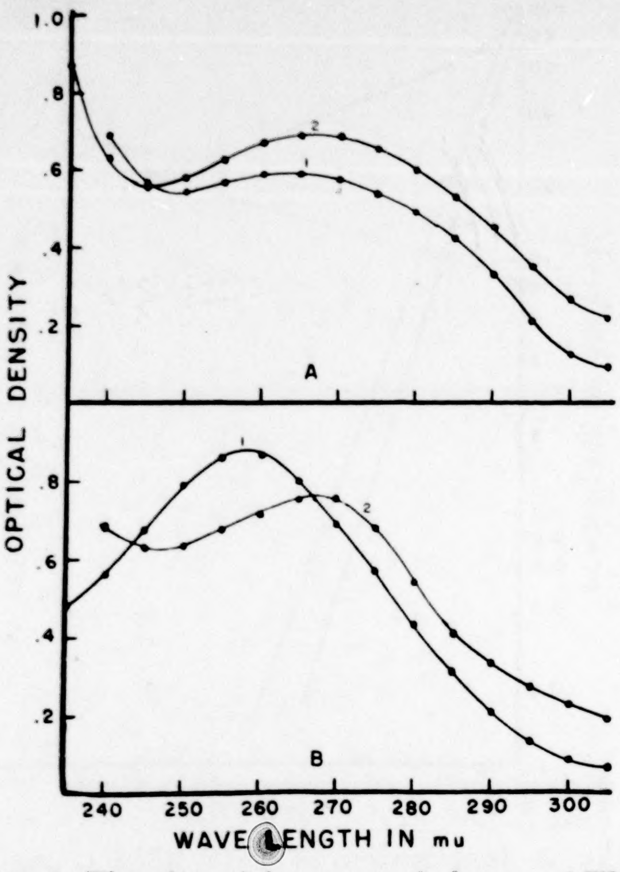

FIG. 9. The ultraviolet spectral change of TMV (A) and TMV RNA (B) after treatment with tannic acid (TA). A. Curve *1*, TMV (200 μg/ml); curve *2*, TMV (200 μg/ml) + TA. B. Curve *1*, TMV RNA (30 μg/ml); curve *2*, TMV RNA (10 μg/ml) + TA.

after reaction with tannic acid. A spectral shift of maximal absorption toward longer wavelengths was noticed in both TMV and TMV RNA treated with tannic acid. These spectra are shown in Fig. 9. The tannic acid-treated TMV was highly diluted, centrifuged, and resuspended in distilled water for the spectral measurement. For TMV RNA, the tannic acid-treated sample was dialyzed against cold 0.02 *M* neutral phosphate buffer for 24 hours. Tannic acid treated TMV RNA sample was also precipitated with 3 volumes of cold ethanol and redissolved in 0.02 *M* acetate buffer at pH 5.5 for the spectral measurement. Therefore, the influence of any free tannic acid on the spectral measurement in these three preparations was negligible. The shift in maximal absorption toward longer wavelengths probably indicates the presence of tannic acid as a complex in combination with TMV or TMV RNA particles.

The attachment of tannic acid to TMV RNA was further demonstrated by the reduced hyperchromic effect (Hall and Doty, 1959) of the absorption spectrum of the complex with rising temperature. TMV RNA in 0.02 *M* acetate buffer (pH 5.5) increased 25% in maximum absorption when the temperature of the solution rose from room temperature to 85°. The increase was almost completely reversible. However, alcohol precipitation of tannic acid-treated TMV RNA to remove free tannic acid caused only 3.4% increase in maximum absorption with temperatures from room temperature to 85°. Such an increase was also reversible.

Reaction of tannic acid with TMV RNA and its purine and pyrimidine components. Upon long standing, tannic acid solution slowly turns brownish. This color also appeared when the solution was neutralized with KOH. Reaction of tannic acid with TMV or TMV RNA accelerated the development of a similar brownish color, being most intense with TMV RNA. A mixture of tannic acid and TMV protein isolated by the glacial acetic acid method of Fraenkel-Conrat (1957) developed the brown color no faster than did tannic acid alone. It seemed, therefore, that the RNA core was responsible for this reaction with tannic acid. Commercial purine and pyrimidine bases and their nucleosides and nucleotides were studied for a reaction with tannic acid. All bases (adenine, guanine, cytosine, uracil, xanthine, isocytosine), nucleosides (adenosine, guanosine, cytosine, uridine), and nucleotides (adenosine 5′-phosphate, guanosine 5′-phosphate, guanosine 2′,3′-phosphate, guanosine triphosphate, cytidine 5′-phosphate, uridine 5′-phosphate), were dissolved in 0.02 *M* acetate buffer at pH 5.5, and tannic acid solution was added to a final concentration of 10^{-3} *M* to each. A strong color developed after 3 days with guanosine 5-phosphate, guanosine 2′,3′-phosphate, guanosine triphosphate or uridine 5′-phosphate, but not with any of the other purine or pyrimidine derivatives tested. Thus, it appears that only 2 of the 4 nucleotides of TMV RNA are reactive with tannic acid, while their respective nucleosides and bases are not.

The reaction product was detected by paper chromatography using isopropanol 2 *N* HCl as solvent. It appeared as an ultraviolet fluorescent streak close to the origin.

The absorption spectrum of this product has a maximum close to 255 mμ with a slight shoulder at 275 mμ when measured in distilled water. Aged, neutralized, or alkaline tannic acid (addition of KOH) produced the same fluorescent streak under ultraviolet light on paper chromatograms developed with isopropanol–2 *N* HCl. The spectrum of this product is similar to the spectrum of phloroglucide, thus it is presumed to be a form of decarboxylated tannic acid.

Paper electrophoresis of the tannic acid-treated uridine 5′-phosphate, guanosine 5′-phosphate, or guanosine triphosphate in both citrate buffer (pH 3.6) and borate buffer (pH 9.0) presented no altered forms of the nucleotides. Two-dimensional paper chromatography was also tried with no detection of new forms.

DISCUSSION

Tannic acid has been reported not only as an inhibitor to tobacco mosaic virus (Thornberry, 1935; Hirth, 1951; Thresh, 1956; Blaszczak *et al.*, 1959), tobacco necrosis virus (Thresh, 1956), and potato virus X (Blaszczak *et al.*, 1959), but also as a bactericidal agent (Floch, 1949; Tsuchihira and Ito, 1951) and as an inhibitor to bacteriophage (Chantrill *et al.*, 1952; Fischer *et al.*, 1954) and influenza virus (Green, 1948; Chantrill *et al.*, 1952; Frisch and Carson, 1953; Carson and Frisch, 1953). As a plant virus inhibitor, it is reported mainly as an exogenous inhibitor, forming a complex with virus particles and preventing the establishment of infection. Working with influenza virus, Green (1948) reported that tannic acid prevented the multiplication of influenza A virus in the allantoic sac when applied up to 6 hours after infection. Frisch and Carson (1953) later indicated that *in vivo* inactivation by tannic acid is temporary and that the virus invariably reappears in the allantoic fluid if sufficient incubation time is permitted. They further suggested that both *in vivo* and *in vitro* inactivation of influenza virus was probably related to the potent protein denaturation by tannic acid.

In our work, tannic acid appeared to react in three different manners with whole TMV, but there was only a single reaction with TMV RNA. At TMV concentrations below 3 mg/ml, tannic acid inactivated 70% of the TMV instantaneously (Fig. 5). This reaction was completely reversible with caffeine. A second reaction leading to increased inactivation seemed to follow first-order kinetics (Fig. 5) and was only partially reversible by caffeine. A third reaction involved increased viscosity and gel formation at concentrations of TMV greater than 3 mg/ml where caffeine was ineffective in restoring activity.

The inactivation of TMV RNA by tannic acid followed first-order kinetics and was completely reversible with caffeine. It would thus appear that tannic acid could possibly inactivate TMV *in vivo* either by reacting with the protein coat or by reacting with the RNA core.

The brownish color that developed in mixtures of tannic acid with either TMV or TMV RNA would imply that even with whole TMV some of the inactivation by tannic acid was caused by its reaction with the RNA core. TMV protein did not produce this reaction. The brownish color appeared to be due to the formation of a decarboxylated reaction product of tannic acid. The accelerated formation of such a product from tannic acid was specific for guanylic and uridylic acids, and it was presumed that these nucleotides were the reactive sites in the RNA. This reaction was of interest because no altered forms of the nucleotides could be detected in a reaction mixture, implying a catalytic type of reaction. If this were true, then it is difficult to explain the loss of infectivity of tannic acid-treated TMV RNA. Since infectivity was restored by caffeine, the bonding of tannic acid to TMV RNA must be a loose one. Changes in ultraviolet absorption spectra of tannic acid-treated TMV RNA would suggest the formation of a tannic acid–RNA complex. It is interesting to note that the hyperchromic response of tannic acid-treated RNA to elevated temperatures was greatly reduced when free tannic acid was removed from the mixture.

In a virus–host system, the *in vitro* effects of tannic acid on TMV or TMV RNA are apt to be mitigated by the complexing of tannic acid by normal host constituents. In the *P. floridana*–TMV system, virus titer

was reduced by tannic acid treatment. It is not clear whether this was due to a direct effect on the virus or to some effect on the host that limited virus synthesis.

In the cucumber–TMV system, several effects of tannic acid were noted. The complete inhibition of starch lesions by tannic acid when applied up to 33 hours after inoculation, with a progressively diminishing effect up to 72 hours, indicated an inhibitory effect on starch lesion development. This inhibitory effect, however, was not on virus replication, but on the host-virus mechanism which prevented starch hydrolysis during a dark period. Thus, tannic acid treatment had no effect on the establishment of infection, but merely suppressed the expression of starch lesions in cucumber.

TMV infection in cucumber is normally limited to an area of about 200 cells around the infection site (Lindner *et al.*, 1959). Tannic acid treatment interfered with the mechanism that limited the infection and permitted systemic development of TMV. The simplest explanation for this effect is to postulate the presence of an interferon-like substance (Loebenstein, 1963) in cucumber that normally limits TMV infection. If tannic acid formed a complex with this substance and prevented its action, then TMV could become systemic. Chick embryo interferon was found to be a protein of low molecular weight (Lampson *et al.*, 1963). It is of interest to note that tannic acid is a good complexing agent for most proteins.

Neither tannic acid nor geranium tannin produced any visible effect on lesion size. Thus, the effects of these materials on the virus localizing mechanism and on starch hydrolysis must have occurred simultaneously, resulting in increased virus replication but with fewer visible lesions.

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Systemic Resistance Induced by Localized Virus Infections: Extent of Changes in Uninfected Plant Parts[1]

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Necrotic lesions caused by tobacco mosaic virus (TMV) in 3-4 lower leaves of a tobacco plant induced resistance to TMV in up to 16 noninoculated leaves; the resistance was characterized by the development of fewer and much smaller lesions in response to subsequent inoculation with TMV than developed in comparably inoculated normal leaves. Similar resistance was not induced by *Pseudomonas tabaci* or by mechanical or chemical injury. No resistance developed unless leaves containing the initial TMV infections remained on the plant for about 3 days. Leaving the initially inoculated leaves on the plant for longer periods resulted in progressively higher levels of resistance up to periods of about 7 days, but delaying the removal for still longer periods resulted in no further increase in resistance. Once a leaf became resistant, it remained so for at least 42 days, the longest period tested.

When the 3 lower leaves of tobacco plants were inoculated with TMV and then removed 7-10 days later, the next 9-16 leaves, but not those above the 16th, became resistant regardless of whether the tested leaves had developed before or after removal of the originally inoculated leaves. Some leaves that became resistant were no more than leaf initials when the leaves containing the original infections were removed.

The respiration rate of disks cut from resistant tissues did not differ significantly from that of disks from normal tissues, nor did resistant and normal tissues differ in quantity of total or individual free amino acids. Polyphenoloxidase activity, as determined manometrically, was essentially the same in extracts from resistant leaves as in extracts of normal leaves.

INTRODUCTION

When stems or a few leaves of tobacco plants hypersensitive to tobacco mosaic virus (TMV) are inoculated with TMV, other parts of the plants become resistant to TMV; subsequent inoculation of other plant parts will result in fewer and smaller lesions than result from similar inoculations of healthy plants (Ross and Bozarth, 1960; Ross, 1961). This resistance phenomenon, termed systemic acquired resistance, occurs in all leaves even though the virus is not translocated beyond the area delimited by the local necrotic lesions (Ross, 1961). This type of resistance has been induced by any of several viruses that cause a necrotic local lesion reaction in tobacco (including tobacco necrosis virus, tobacco ringspot virus, tomato ringspot virus, and turnip mosaic virus), but not by viruses that invade tobacco systemically without inducing local necrosis (Ross, 1961). A similar, if not identical, reaction is also induced by the fungus *Thielaviopsis basicola* (Berk. & Br.) Ferr., which causes necrotic lesions in tobacco similar to those induced by tobacco necrosis virus (Bozarth *et al.*, 1962; Hecht and Bateman, 1964). The resistance has not been induced by mechanical abrasion or chemically induced necrosis (Ross, 1961).

The objectives of the present work were

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to test other nonviral agents for ability to induce resistance to TMV; to determine how long the initial TMV-infected leaves must remain on the plant to induce detectable, as well as maximal, resistance in other plant parts; to determine the persistence of the induced resistance; to determine the extent to which localized infections cause the development of resistance in all other leaves in large plants and in leaves formed subsequent to removal of the initial infections; and to determine the effect of resistance on respiration, polyphenoloxidase activity, and content of free amino acids.

MATERIALS AND METHODS

In general, the materials and methods used were similar to those used by Ross (1961). Except where stated otherwise, the test plant used was *Nicotiana tabacum* L. var. Samsun NN which contains the *N* gene from *N. glutinosa* L. (Holmes, 1938). The plants were grown in steam-sterilized composted soil in 4-inch pots in a greenhouse and given supplemental fertilizer.

The principal tests used were the half-leaf tests, in which one half of each leaf on trimmed tobacco plants (2 leaves) was first inoculated with TMV and the opposite half subsequently challenge inoculated with TMV, and multiple-leaf tests, in which 1–3 leaves of each test plant were first inoculated with TMV and the other leaves of the same plant challenge inoculated with TMV at a later date. Control plants were rubbed with water in the first inoculation. At least 5 plants were used for each treatment. Lesion diameters were measured with a stereoscopic microscope equipped with an ocular micrometer; generally, at least 5 randomly chosen lesions from each leaf used were measured 7 days after the second inoculation.

The common strain of TMV was used for all inoculations. Inocula consisted of clarified juice of systemically infected Turkish tobacco plants (*N. tabacum*); the juice was divided into small portions that were kept frozen until used, when they were thawed and diluted 1:1000 with distilled water, except where stated otherwise.

The culture of *Pseudomonas tabaci* (Wolf & Foster) Stapp was obtained from the American Type Culture Collection and maintained on potato dextrose agar. It was transferred to fresh media 5 days prior to inoculation of tobacco leaves by rubbing a suspension of bacteria on the leaves. Carborundum was used as an abrasive.

RESULTS

Attempts to Induce Resistance by Agents Other Than Viruses

Although Ross (1961) was unable to induce resistance by mechanical or chemical injury, the accumulation of data indicating an almost complete lack of virus specificity in the resistance reaction indicated need for reinvestigation of this question. Consequently, additional means of inducing necrosis were tested as possible inducers of resistance.

In 2 experiments, the 3 lower leaves of a group of plants trimmed to 4 leaves were injured by placing 75–100 drops of concentrated H_2SO_4 on each leaf. Another group was treated similarly with concentrated HCl. A third treatment consisted of the usual TMV inoculation. A fourth treatment involved pressing a wire brush to the upper surface of each leaf. The upper leaf on each plant was challenge inoculated 7 days later with TMV.

Although the methods used to simulate virus-induced necrotic lesions resulted in numerous necrotic spots in both experiments, resistance developed only in those plants inoculated with TMV. Other treatments that caused severe injury to lower leaves without inducing resistance in upper leaves were: spraying with dilute $CuSO_4$, rubbing with coarse sand, and rubbing with coarse sand plus potato virus Y. The leaf-spotting bacterium, *Pseudomonas tabaci*, also failed to induce resistance in 2 tests similar to the above. In parallel tests, inoculation of lower leaves with TMV always induced good resistance, i.e., lesions in test plants had diameters $\frac{1}{4}$–$\frac{1}{5}$ as great as those of lesions in control plants.

Rate of Development and Persistence of Resistance

Ross (1961) reported on the rate at which systemic induced resistance develops and on how long it persists; there was no abrupt cutoff of the resistance-inducing principle by

removal of the first-inoculated leaves before challenge inoculation. Experiments were therefore made to determine the rate of development of resistance and its persistence when the first-inoculated leaves or leaf-halves are removed at intervals after the first inoculation but before challenge inoculation.

The apical halves of the leaves of a large group of trimmed tobacco plants were inoculated with TMV and were removed 3, 4, 6, 8, or 10 days later. Ten days after the first inoculation, the basal halves of all leaves were challenge inoculated. Lesion size in the test plants decreased progressively as the interval between the first inoculation and leaf removal was increased up to 8 days. The ratios of mean diameter of lesions in protected plants to those of lesions in corresponding control plants were 0.67, 0.55, 0.40, 0.30, and 0.33 when the intervals were 3, 4, 6, 8, and 10 days, respectively. Virtually identical results were obtained with Xanthi-nc tobacco [another variety containing the *N* gene and thus hypersensitive to TMV (Takahashi, 1956)]. In a 2-leaf test in which the lower inoculated leaves were removed after 1, 2, 3, 4, 5, 6, or 7 days, challenge inoculation on the seventh day resulted in no detectable resistance when the leaves were removed in 1 day, slight resistance when they were removed in 2–3 days, and highest resistance when they were removed in 6 days.

In a further test, resistance was not induced within 3 days, it was not increased by leaving the first-inoculated leaves on the plants for periods longer than a week, and upper leaves remained resistant for at least 17 days after removal of the inciting infections (Table 1).

TABLE 1

Level of Resistance Developing and Persisting in 2 Upper Leaves on Samsum NN Tobacco Plants, 3 Lower Leaves of Which Were Inoculated with TMV and Left on the Plants for Specific Periods

Days lower leaves were on plant after day 0	Diameter of lesions[a] in upper leaves inoculated on day 21		
	I (mm)	C (mm)	(I/C)
0	2.00	2.08	0.96
1	1.74	1.82	0.96
2	1.92	1.74	1.10
3	1.89	2.04	0.93
4	0.70	1.92	0.36
5	0.63	2.29	0.28
7	0.50	2.05	0.24
9	0.52	1.76	0.30
12	0.54	2.20	0.25
15	0.48	1.72	0.28
21	0.50	1.68	0.30
28[b]	0.47	1.98	0.24

[a] Each mean based on 100 lesions (2 diameters per lesion measured on day 28) in 5 plants, 2 leaves per plant. "I" indicates inoculated series; "C" indicates control series, the lower leaves of which were removed at the same time as those of the I series.

[b] Lower leaves not removed.

Development and Distribution of Resistance in Large Untrimmed Plants

In earlier tests Ross (1961) found that the age of the leaf or half-leaf challenged had no consistent effect on the level of resistance that developed in it. He found that resistant leaves developed from the first several axillary buds emerging after the inducing inoculation of decapitated plants, but other leaves developing several weeks later from axillary buds were fully susceptible. An explanation for this anomaly was sought in experiments with large untrimmed plants.

Thirty small Samsun NN tobacco plants selected for uniformity were transplanted to 6-inch pots; half of these plants were given 1.5 g NH_4NO_3 per pot. They were inoculated on the 3 lower leaves when they had 9 well-expanded leaves. Five plants of each group were inoculated with infectious juice diluted 10^{-3}, and 5 plants with infectious juice diluted 10^{-5}; 5 noninoculated plants were maintained as controls. About 300–500 lesions developed in each leaf inoculated with 10^{-3} inoculum and 30–35 lesions in leaves inoculated with the 10^{-5} inoculum.

Seven days after the inducing inoculation all plants were decapitated so that the uppermost leaves would expand. By this time, those plants not receiving extra nitrogen were pale green and had only 6 usable leaves as compared to 9 for the plants given extra nitrogen. Two weeks after the first inoculation, all leaves of all plants except those leaves previously inoculated (or comparable

leaves on the control plants) were challenge inoculated with TMV.

In the well-fertilized series, all upper leaves became resistant, although the more potent inoculum consistently induced a higher level of resistance than did the dilute inoculum (Fig. 1). The lowest 6 leaves became highly resistant and the uppermost 3 less so. In general, there was a progressive increase in lesion size at each successively higher leaf position on both control and inoculated plants, an indication that an increase in age of leaf results in development of resistance similar to that induced by localized infections.

The results in the low-nitrogen series were similar to those in the other; except that the more dilute inoculum failed to induce resistance in the uppermost leaves.

In another experiment with Xanthi-nc tobacco plants having 28 leaves, the lower 4 leaves were inoculated and removed 10 days later at the time of the challenge inoculation. Resistance developed in 16 of the remaining leaves; the lowest 6 developed high resistance and the next 10 progressively less. The uppermost leaves remained fully susceptible.

That the youngest leaves of tall plants are capable of developing resistance was shown by results of a half-leaf test with Xanthi-nc plants of the same size as those used in the previous experiment. The left half of each leaf was inoculated with TMV, and the right half was challenge inoculated with TMV 7 days later. Resistance developed in the right half of all leaves, but highest resistance developed in the lowest 3 leaves, where lesions in protected leaf halves had diameters 20% as great as those of lesions in control leaves, and least resistance developed in the uppermost 3 leaves, where lesion diameter in test leaves was 44% as great as those of lesions in control leaves.

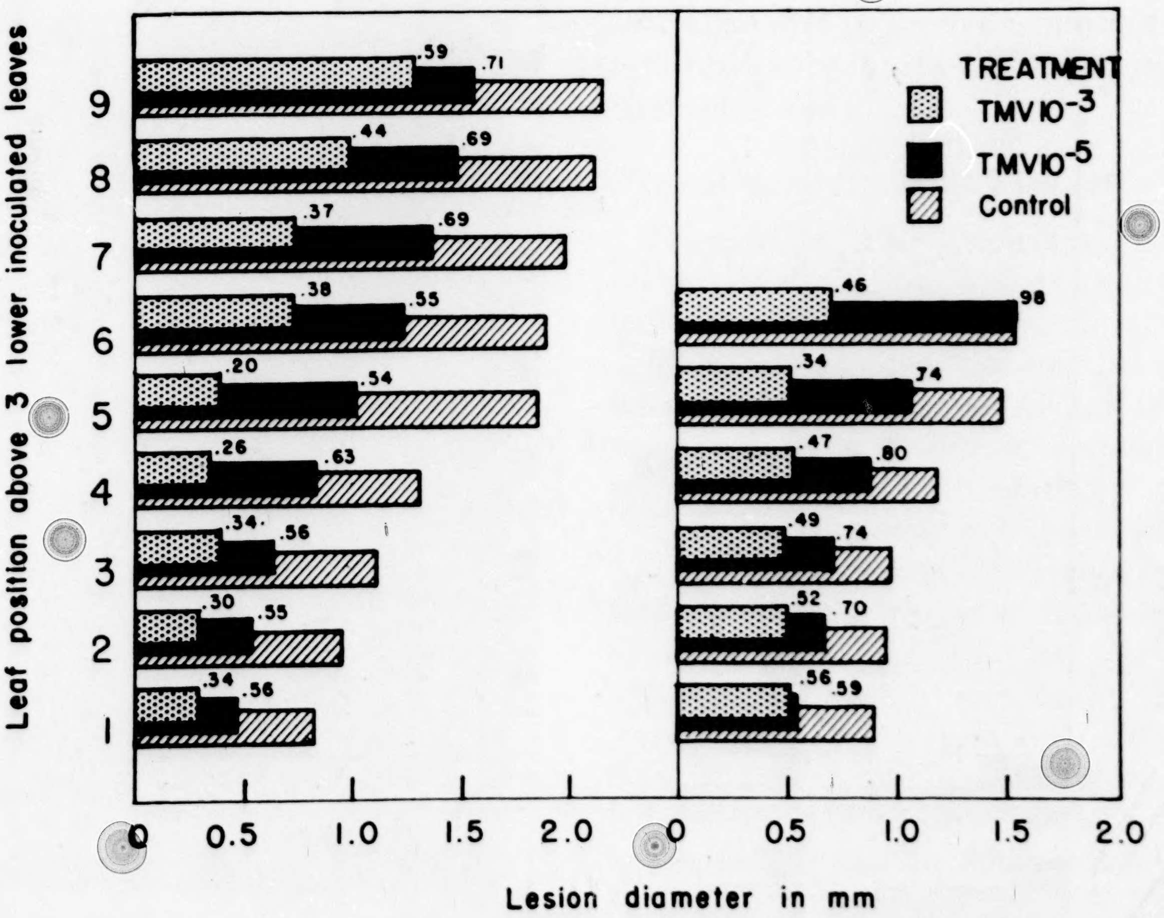

FIG. 1. Development of induced resistance at each leaf position of large Samsun NN tobacco plants following inoculation of the 3 lower leaves with TMV. For this inoculation, infectious juice diluted 10^{-3} was used for 1 series of plants and juice diluted 10^{-5} for another. Plants were decapitated after 1 week and challenge inoculated with TMV 1 week later. Mean diameters for each treatment are based on 25 lesions measured in leaves from 5 plants 1 week after challenge inoculation. Numerals over the bars indicate the ratio of lesion diameter in test plants to that of lesions in corresponding control plants. Left: Plants grown at high nitrogen level (1.5 g supplemental NH_4NO_3 per pot). Right: no supplemental nitrogen. (Photograph by H. H. Lyon.)

Resistance Induced in Leaves Produced Subsequent to Inoculation of Lower Leaves

Data obtained in the experiments with tall plants indicated that localized infections by TMV in a few leaves on such plants will induce resistance in many, but not necessarily all, of the leaves on the plants at the time of inoculation. This raised the question whether similar patterns of distribution of resistance would occur in plants allowed to develop most of their leaves from the apical growing tip subsequent to the inducing inoculation. Somewhat similar patterns were anticipated on the basis of earlier results (Ross, 1961) with leaves developing from axillary buds following decapitation.

Half of a large group of small NN tobacco plants, each with 3 leaves 6–8 cm in length, were inoculated with TMV; the inoculated leaves were removed 10 days later. At 4 weekly intervals, beginning 1 week after the

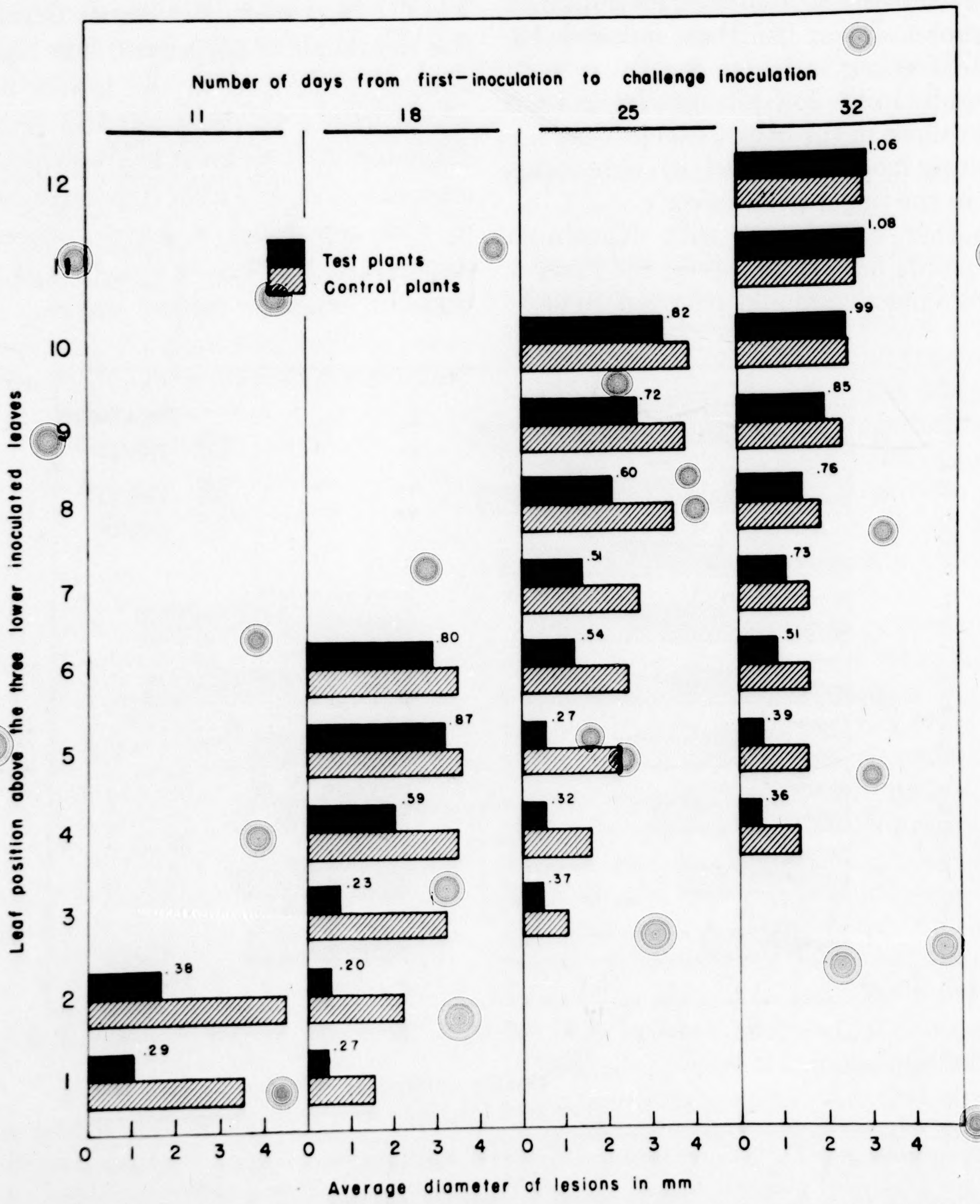

FIG. 2. Development of induced resistance in upper leaves in Samsun NN tobacco plants inoculated (3 leaves) with TMV when young and allowed to develop different numbers of leaves before challenge inoculation with TMV. Numbers above bars indicate the ratio of lesion diameter in test leaves to that in control leaves. Averages are based on measurements, made 1 week after challenge inoculation, of 25 lesions per leaf (6 plants per treatment). (Photograph by H. H. Lyon.)

first inoculation, 6 previously inoculated and 6 control plants were decapitated above the highest reasonably well-expanded leaf; 4 days afterward, all leaves present were challenge inoculated with TMV.

Again, lesion size was correlated with leaf maturity in both the previously inoculated and the control series (Figs. 2 and 3). With each challenge inoculation, there was an increase in lesion size from the lower to upper leaves. Also, there was a progressive decrease in lesion size at any given leaf position from the first through the last inoculation date. Thus the relation between lesion size and leaf maturity, observed in the previous experiment, was confirmed in two different ways.

In general the highest level of resistance was induced in leaves nearest the first-inoculated leaves. The level of resistance tended to be progressively less at each successively higher leaf position until eventually a point was reached where leaves on test plants were as susceptible as those on control plants (Fig. 2). Some of the leaves, developing from the growing tip after removal of the infected lower leaves, became resistant even though their expansion had not begun until after the resistance-inducing infections had been removed.

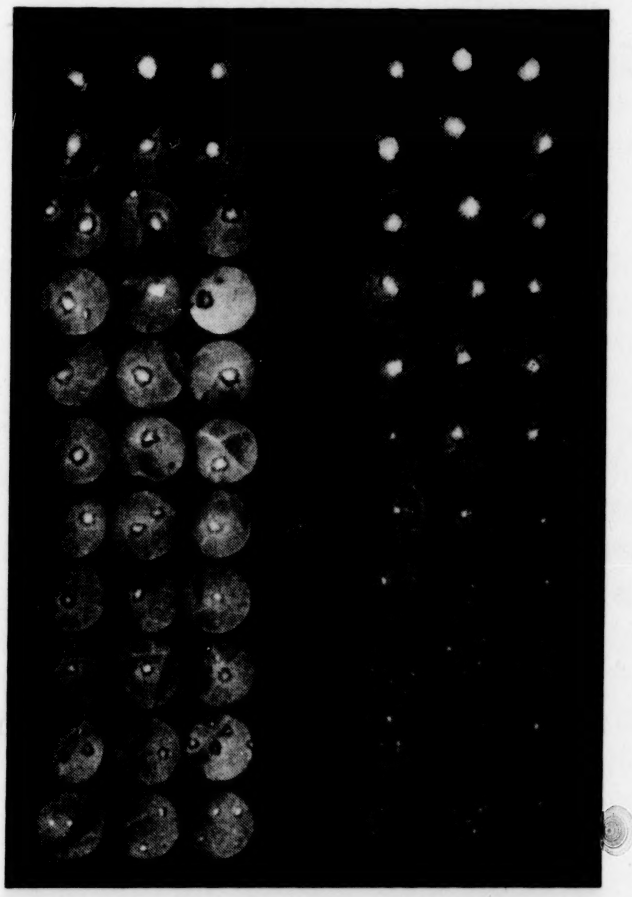

FIG. 3. TMV lesions in disks cut from successively higher leaves of Samsun NN tobacco 7 days after challenge inoculation. Right: Lesions in upper leaves from plants that were first inoculated when very young (3 leaves inoculated with TMV); upper leaves were challenge inoculated 32 days later. Left: Lesions resulting from challenge inoculation of control plants which did not receive the first inoculation. (Photograph by H. H. Lyon.)

Since the results of the previous experiment raised the question whether similar distribution patterns would result when challenge inoculation was delayed still longer, the experiment was repeated with a single challenge inoculation made 49 days after the first inoculation, when each plant had 23 usable leaves. Again, lesion size increased progressively from bottom to top. The lowest leaf had only slight resistance, the next 4 were highly resistant, and successively higher leaves were progressively less resistant up to the 15th or 16th leaf (Fig. 4). These results differ from those of the previous experiment chiefly in that the lowest leaf appeared to have declined in resistance and resistance appeared further up the stem than previously.

Physiology of Resistant Tissues

Respiration. The respiration rate of leaf disks punched from resistant and control half-leaves on the first, fourth, and seventh day, following inoculation or simulated inoculation of the opposite half-leaves, was measured in a Warburg constant-volume respirometer at 30°C (±0.05°).

The mean quantity of oxygen absorbed by 15 disks (0.12 g) was 5% higher in the control than in the resistant half-leaves at all times of sampling; however, the difference was not statistically significant at the 5% level except on the first day after inoculation. It seems unlikely, therefore, that resistant and control leaves differ appreciably in respiration rate at times when resistance is at its highest level.

Polyphenoloxidase activity. Because of the importance placed by some workers (Solymosy *et al.*, 1959) on polyphenoloxidase activity in lesion development, an extensive series of experiments was made to determine whether there was any correlation between polyphenoloxidase activity and the development of systemic induced resistance.

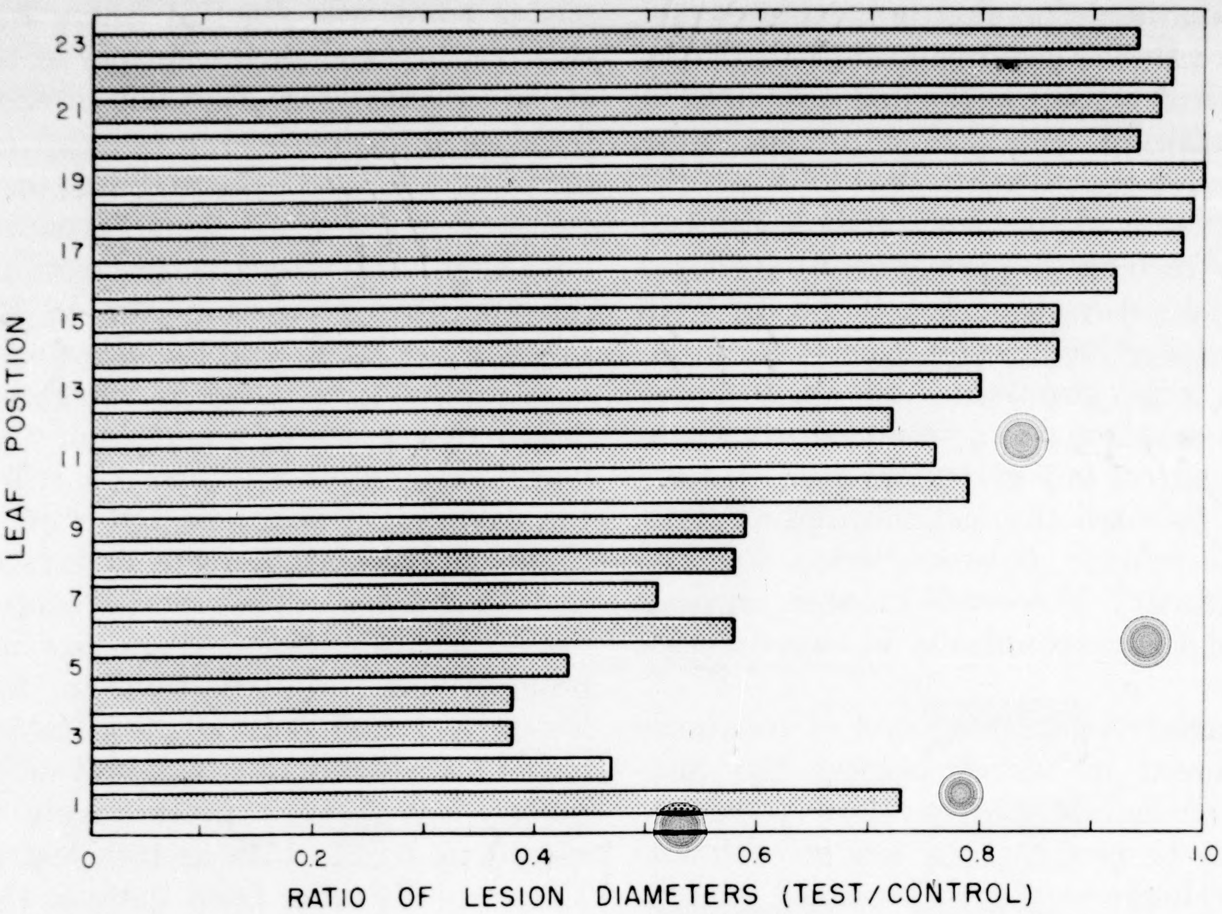

FIG. 4. Development of induced resistance, as indicated by ratio of lesion diameter in test leaves to that in control leaves, in upper leaves of Samsun NN tobacco plants inoculated (3 leaves) when young with TMV and allowed to develop 23 leaves before challenge inoculation of upper leaves with TMV 49 days after the first inoculation. The first-inoculated leaves were removed 7 days after inoculation. Means are based on measurements (2 diameters per lesion) of 100 lesions (from 6 plants) from each leaf position. (Photograph by W. G. Smith.)

Polyphenoloxidase activity was assayed by the manometric method of Goddard and Holden (1950). Juice from resistant and control leaves was extracted at 2° by grinding leaves with a mortar and pestle in 0.1 *M* potassium phosphate buffer pH 6.5 and centrifuging at 3000 *g*. The clear supernatant solution was immediately tested for polyphenoloxidase activity, hydroquinone and catechol being used as substrates.

Twenty experiments were made with resistant leaves and control leaves. In 4 experiments, the polyphenoloxidase activity was virtually the same in both kinds of leaves. The resistant leaves had the higher activity in 6 experiments, and the control leaves had the higher polyphenoloxidase activity in 10 experiments.

Throughout the experiments on polyphenoloxidase activity, there was much variability in the amount of O_2 absorbed in different experiments with apparently uniform material. Experiments were initiated to define the sources of variation. When plants were trimmed to 4 leaves and each leaf was assayed for polyphenoloxidase activity several days later, there was an increase in activity from the bottom to the top leaf. In 2 experiments, the ratios from bottom to top leaves were 6:6:11:14 and 6:7:20:21.

There was no definite correlation between time of day when leaves were harvested and polyphenoloxidase activity in either resistant or control leaves, although control leaves tended to have a greater activity than did resistant ones when harvested in the morning.

Plant variability, sampling error, or some other factor must account for the fact that ratios of enzyme activity of resistant to those of control leaves ranged from 0.5 to 2.3 in these experiments. When the results of 17 experiments in which polyphenoloxidase activity was measured in resistant and control upper leaves were averaged, it was found that there was only a 2% difference in the means of the two treatments. Since there

was a direct correlation between leaf position and polyphenoloxidase activity and a similar correlation between leaf position and lesion size in control plants in other experiments, it was concluded that this enzyme is not a major factor in the development of resistance—at least not the measurable polyphenoloxidase activity prior to the time of the challenge inoculation.

Free amino acids and amides. The free amino acids and amides of resistant and control half-leaves were quantitatively determined 1, 4, and 7 days after the first inoculation. Plants were grown in a growth chamber at 20° under continuous light of about 500–600 foot-candles. The free amino acids were extracted with 80% ethanol, and the amino acids and amides were determined by the quantitative paper chromatography method of Thompson *et al.* (1951). All extractions were made in triplicate, chromatograms were run in duplicate, and the experiment was repeated 3 times. The data were subjected to analysis of variance. In no case was there a qualitative or quantitative difference between the free amino acids of protected and nonprotected tissues.

DISCUSSION

The type of induced resistance dealt with here appears to be associated with any necrosis resulting from a continued irritation of plant tissue possessing an effective mechanism for limiting the extent of the necrosis. In other words, highest levels of resistance develop where the necrotic lesions induced by a pathogen enlarge for a few days and then cease to do so. Thus, little or no resistance results from necrosis induced by mechanical or chemical injury, from infection by non-necrosis-inducing viruses, or by viruses or bacteria that induce necroses that do not become sharply delimited. The accumulated data seem to indicate that the mechanism involved in this type of induced resistance is no more than an activation or intensification of the plant's normal defense reactions, i.e., the reactions that normally limit the extent of invasion of the host by a pathogen to which it is hypersensitive. The data obtained to date are consistent with the hypothesis that a pathogen multiplying in a hypersensitive host, or the resulting necrotic process, results in the production of a material capable of moving systemically and of altering the reaction of the invaded tissues to subsequent inoculation with the same pathogen or others inducing a similar host reaction. The above ideas are compatible if the substance produced is considered to stimulate the host's defense mechanism rather than to inhibit the virus directly.

The fact that leaves developing long after removal of the inciting infections become resistant indicates that resistance is not the result of metabolites moving from noninfected to infected leaves. Instead, it appears that a change resulting in resistance either can occur before the leaf initial begins to develop or that some of the resistance-inducing substance translocated to leaves existing at the time of the initial virus multiplication is later retranslocated to newly developed leaves. Once tissues become resistant, they remain resistant for long periods; it is not yet clear, however, whether this results simply from persistence of the resistance-inducing factor in the tissues or whether the tissues become altered. The distribution pattern of induced resistance in plants allowed to develop many leaves after removal of the inciting infections (Fig. 4) is the only evidence that the induced resistance may not be permanent. Interpretation of these data as indicating that the resistance-inducing substance can move into a leaf and thence to another leaf is consistent with the report by Canadian workers (Gilpatrick and Weintraub, 1952; Weintraub and Kemp, 1961) of the graft transmission of a resistance-inducing factor from dianthus plants possessing virus-induced resistance similar to that developing in TMV-infected Samsun NN tobacco.

In general, the results of experiments in which the initially inoculated leaves were removed at intervals are almost identical with those of tests in which the first-inoculated leaves were not removed (Ross, 1961). This supports the earlier conclusion that the stimulus that induces resistance is not produced after about 7 days and that the effect of this stimulus appears to be permanent. If resistance is induced by a substance moving from the inoculated leaves to other leaves, the results also suggest that this ma-

terial, to be effective, must reach the test leaves before the challenge inoculation is made.

The nature of the assumed resistance-inducing material is not known. Many attempts by Ross (1961) and by Bozarth (1962) to extract such a material were unsuccessful. A possible indication of its nature may be provided by the work of Loebenstein and Ross (1963), who reported extraction of an inhibitor of infection from apical halves of leaves of *Datura stramonium* L. whose basal halves had previously been inoculated with TMV. The inhibitor appeared to be a protein. There is no direct evidence, however, that that material is involved in the resistance phenomenon. Its only known biological property is its ability to inhibit infection when mixed with inocula; it is not known whether or not it affects events occurring subsequent to the establishment of infection, i.e., lesion size. In contrast, the available evidence indicates that the primary effect of the resistance induced by localized infections in hypersensitive hosts is on lesion size, not on the number of lesions (Ross, 1961). Analogies between the supposed resistance-inducing substance and interferon have been suggested (Loebenstein, 1963; Loebenstein and Ross, 1963); such analogies have little support in view of the paucity of data concerning the nature of the resistance-inducing factor and of the huge differences between the tissues and systems of plants and vertebrate animals. A better analogy can be drawn between the resistance factor and phytoalexins as described by Müller (1958) and further investigated by Cruickshank and Perrin (1961). If the suggested resistance-inducing substance exists, it would appear to possess the properties listed by Cruickshank (1963) as characteristic of materials classed as phytoalexins.

Since this work was completed, van Kammen and Brouwer (1964) have reported data seeming in disagreement with ours on polyphenoloxidase activity in resistant tissues. They inoculated portions of Samsun NN leaves with TMV and found a significant increase in polyphenoloxidase activity in the inoculated portions. The increase began when the local lesions appeared and reached a maximum (as much as twice that in control leaves) in 4 days, after which it decreased. Apical halves were inoculated and then removed at intervals; enzyme assays made 4 days after the original inoculation showed that the change resulting in increased polyphenoloxidase activity could be induced in as short a time as 7 hours, i.e., the increase occurred when the infected apical halves were removed after only 7 hours. This difference in results may be due to differences in methodology; van Kammen and Brouwer subjected their extracts to low speed centrifugation and dialysis, whereas we did not. Also, they employed a different substrate and used a spectrophotometric method for determining enzyme activity. Even if there is an actual increase in polyphenoloxidase activity in uninfected parts following inoculation of other parts, it apparently plays no major role in the resistance phenomenon. Our data show that a contact period of 7 hours between the infected and uninfected portions of a leaf is insufficient to induce resistance. In fact, a high level of resistance has never been induced in any kind of test in less than 3 days. Also, the data on polyphenoloxidase activity in different leaves on tall plants show that marked restriction of lesion size is not necessarily correlated with high polyphenoloxidase activity.

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Electron Microscopic Demonstration of Viruslike Particles in *Peregrinus maidis* following Acquisition of Maize Mosaic Virus

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Electron microscopic investigations revealed viruslike particles in salivary glands and epithelial cells of intestine wall of infective *Peregrinus maidis* (Ashm.) following acquisition of maize mosaic virus I (Smith). These particles appeared identical with the ones previously observed in electron micrographs of sections of infected plants. In salivary glands the particles were found in tubules and cisterns of the endoplasmic reticulum and in vacuolelike structures. In intestine cells the particles were seen in the perinuclear space and in tubules. The distribution of the particles indicates that they may develop in the endoplasmic reticulum. It is assumed that the particles are the viral agent of maize mosaic virus I.

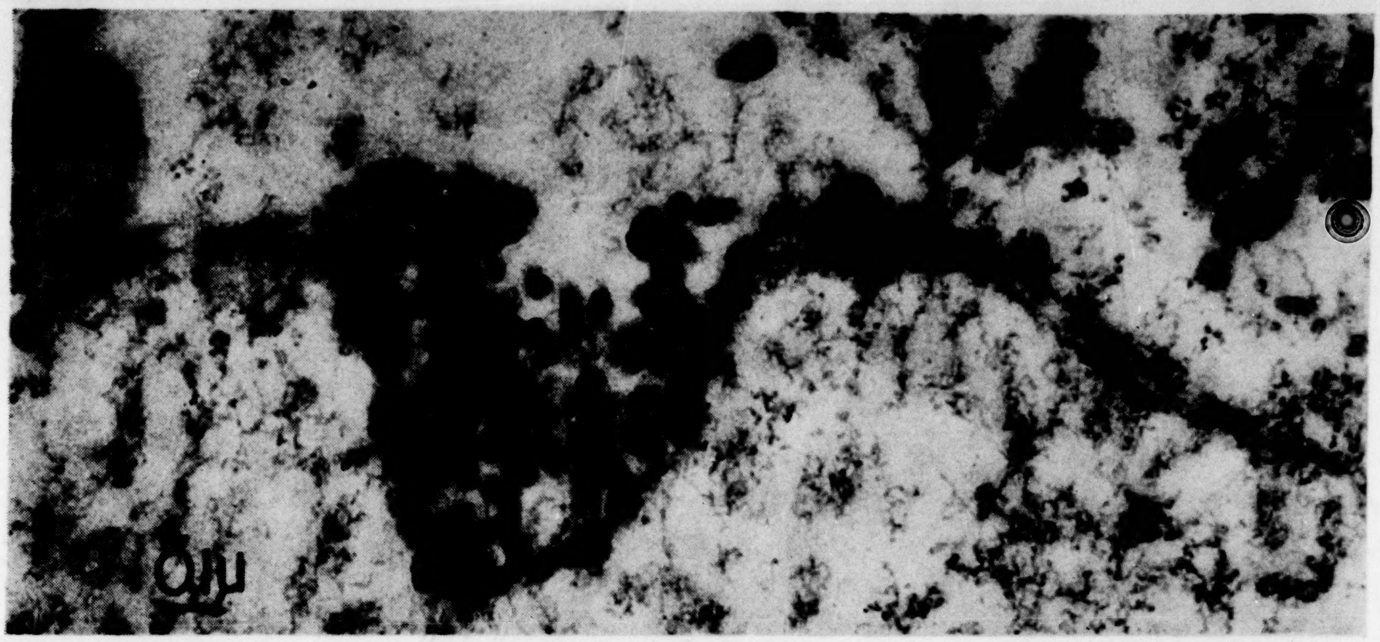
INTRODUCTION

Particles believed to be viral agents were found in diseased corn plants (*Zea mays* L.) (Herold *et al.*, 1960). The causal agent of the disease was transmitted by *Peregrinus maidis* (Ashm.) (Herold, 1963), which is known only as vector of maize mosaic virus I (Smith), (Carter, 1941; Klinkowski, 1954; Smith, 1957). For this reason and because the symptoms of the corn disease (Herold *et al.*, 1960; Malaguti, 1963) correspond to those of maize mosaic virus I, the disease is considered to be caused by maize mosaic virus I (Herold, 1963).

The virus has a long incubation period (9–58 days) in *P. maidis*. It was suspected that during this time the virus multiplied in the insect and could be found in the salivary glands and probably the intestine wall. In the case of two other plant-insect viruses (Fukushi *et al.*, 1962; Shikata *et al.*, 1964) virus particles have been demonstrated both in host plants and insect vectors.

MATERIALS AND METHODS

P. maidis were fed for 3–4 days on infected corn plants, then transferred for 10–12 days to healthy corn plants, and finally kept for 3–4 days singly on other healthy corn plants. Then one by one the insects were killed by chloroform vapors and immediately dissected in a drop of Veronal-buffered 1% osmic acid (pH 7.5). The two largest parts of the compound salivary gland and the intestine were separated. These samples were fixed for 3 hours in osmic acid at 0–4°C, partially dehydrated in graded ethanol and kept in 80% ethanol at 4° for 6–8 weeks. This waiting period served to determine which insects had transmitted virus (infective insects), thus saving unnecessary embedding of samples, because only about 5% of the insects were infective (Herold, 1963). Salivary glands and intestines of infective and some of the noninfective insects that had fed upon diseased plants were dehydrated and embedded in butyl-methyl methacrylate 7:3. Insects reared on healthy plants and never exposed to virus were treated in the same manner as controls. All preparations were sectioned with a diamond knife in a Morán-type microtome (Fernández-Morán, 1956). The sections were stained for 30 minutes in 6% uranyl acetate (Wat-



FIGS. 1 and 2. Sections of salivary glands of *Peregrinus maidis* showing groups of many virus particles in vacuolelike structures. The particles are cut longitudinally, obliquely, and transversally.

son, 1958) and examined in a Siemens Elmiskop I.

RESULTS

Elongated particles of characteristic form and size were found in the salivary glands and cells of intestine wall of infective *P. maidis*. In the salivary glands, the particles were found only in the peripheral parts of the lobes. In the intestine, the particles were at the posterior end of the mid-intestine in epithelial cells having microvilli. The particles seen were cut transversally, longitudinally, and obliquely. They occurred singly and in groups of few or many. The particles found in the salivary glands were always in tubules (Figs. 3A, double arrows; 3C) and cisterns (Fig. 3A, single arrows) of the endoplasmic reticulum and in vacuolelike structures (Figs. 1 and 2). Several particles were positioned at right angles to the major axis of the cisterns (Fig. 3A, single arrows),

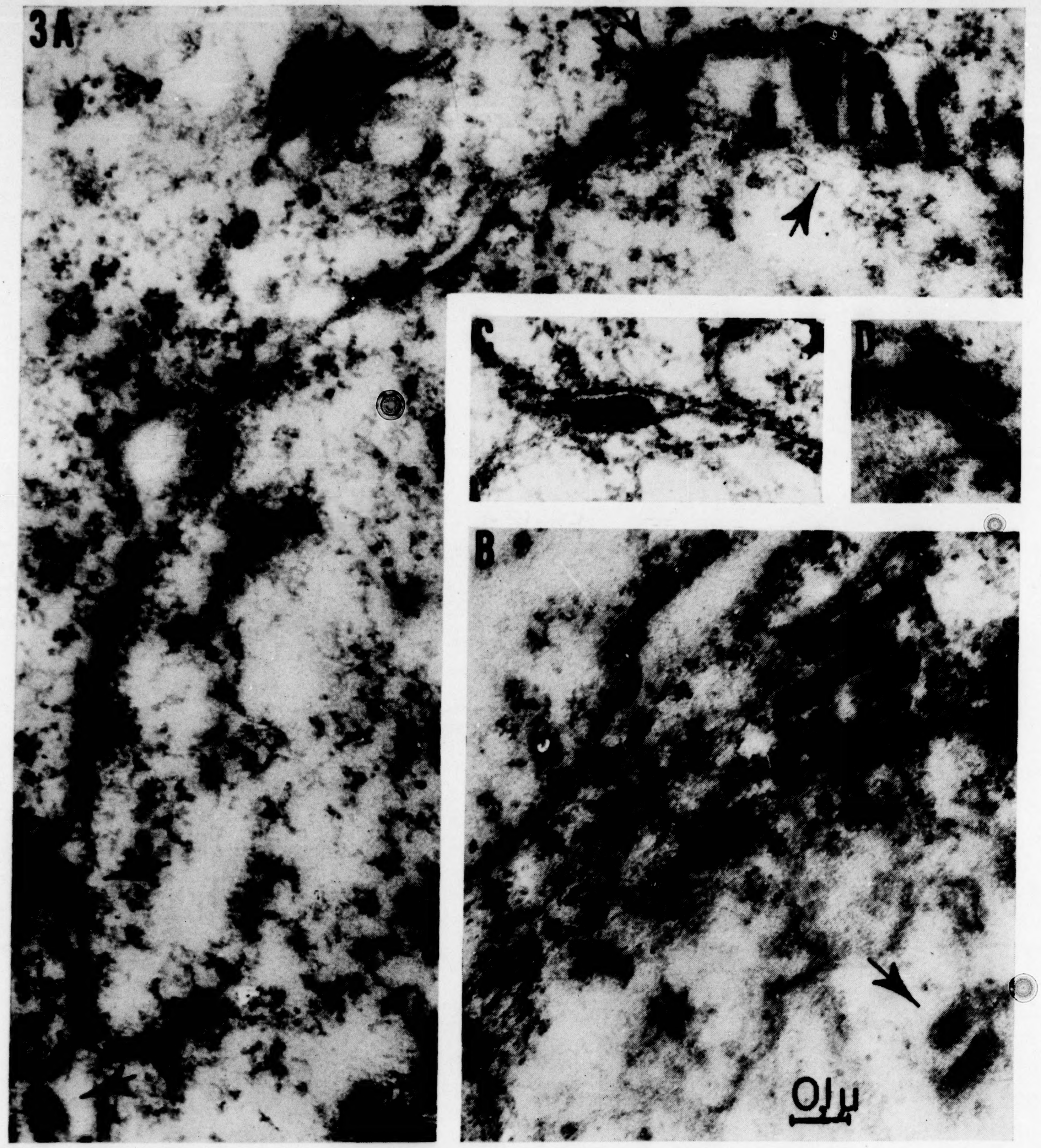


FIG. 3. Sections of salivary glands showing groups of few and single virus particles in the endoplasmic reticulum. In Figs. 3A, B, and C, the particles are seen in cisterns and tubules; in A, some groups appear transversally in the cistern (single arrows) and one particle longitudinally in the wider part of the tubule (double arrows); in C the particle lies longitudinally in the tubule. Some particles in B (arrow), and those in C and D demonstrate two limiting membranes and a central core. In B at the lower left three particles appear cross sectioned.

others lay parallel to the major axis of the tubules (Figs. 3A, double arrow; 3C). In the intestine cells, the particles were found in the perinuclear space perpendicular to the nuclear surface (Figs. 4, single arrows, and 5) and in tubules of the endoplasmic reticulum. In some particles one or two limiting membranes were observed and in some an electron dense core was seen (Figs. 3B, arrow, 3C, 3D, and 5). The average length of particles was 224 mμ and the average diameter was 50 mμ. The particles were found in 4 of 12 glands and in 1 of 2 intestines from infective *P. maidis*. No particles were

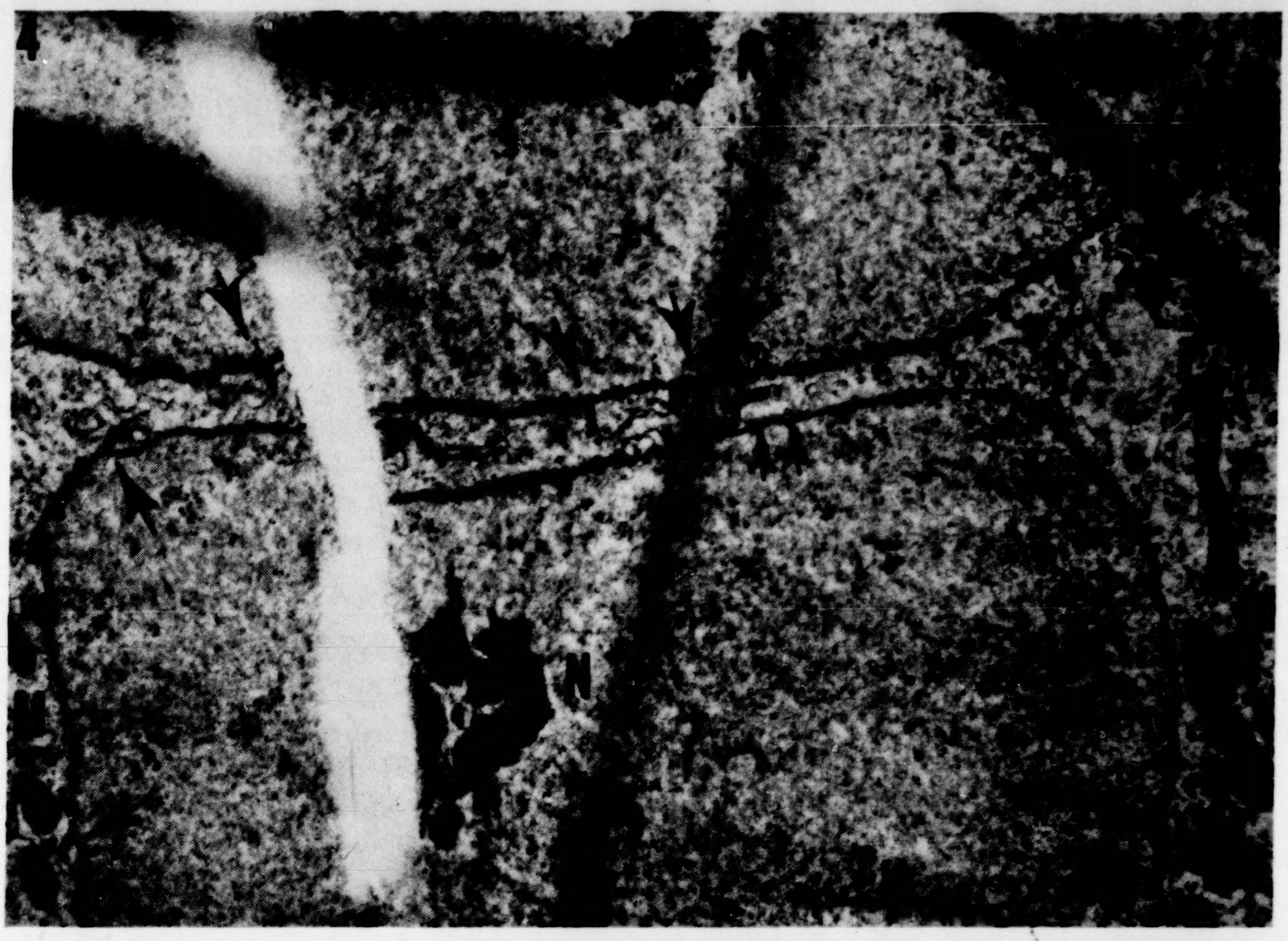


FIG. 4. Section of an epithelial cell of the intestine wall of *Peregrinus maidis* showing several virus particles in the perinuclear space positioned perpendicular to the nuclear surface (single arrows). Other particles are seen in tubules cut longitudinally (double arrows) or cut transversally (triple arrows); *NM*, nuclear membranes; *N*, nucleolus; *M*, mitochondrion.

FIG. 5. Section of an epithelial cell of the intestine wall at higher magnification. Virus particles are seen in the perinuclear space cut longitudinally and in tubules of the endoplasmic reticulum cut longitudinally and transversally. In some particles two limiting membranes and a central core can be seen. Fig. 5B is an enlargement of Fig. 4 (center).

found in 17 glands and 9 intestines of healthy insects of laboratory rearings and in 12 glands and 5 intestines of insects that had fed upon diseased plants but did not transmit virus.

DISCUSSION

The particles are considered to be agents of maize mosaic virus I because (1) they were only found in infective *P. maidis*; (2) they had a characteristic morphology with 2

membranes and an inside core described in particles of corn plants infected with maize mosaic virus I (Herold *et al.*, 1960); and (3) they were only slightly shorter than those of maize mosaic virus I. The shorter length found in the present investigation was based only on 18 particles and may therefore not be significant.

It is surprising that the virus like particles were found in only 4 of 12 salivary glands of infective *P. maidis*. However, the particles were unevenly distributed in the cells and were present only in low concentration. Carter's observations (1941) that infective *P. maidis* did not transmit on all days could also be interpreted to mean that the virus was present in low concentration.

It is thought that the vacuolelike structures surrounding some groups of viruslike particles (Figs. 1 and 2) were originally cisterns, because of the continuation of the membranes and their structural similarities with the endoplasmic reticulum.

The viruslike particles seem to move in the lumen of the tubules of the endoplasmic reticulum, since they are found occasionally at right angles to the major axis of the tubules. Such a position could have been the result of being caught at one end and consequently being locked in this position. It is assumed that the viruses are formed at the inner wall of the endoplasmic reticulum and the perinuclear space. The latter can be considered as a differentiation of the endoplasmic reticulum. The development of virus inside of vesicles of the endoplasmic reticulum was first suggested by Bergold and Weibel (1962) with yellow fever virus. Mussgay and Weibel (1963) observed maturing of vesicular stomatitis virus at cytoplasmic membranes. Howatson and Whitmore (1962) described development of vesicular stomatitis virus, and Berkaloff (1963) showed formation of Sendaï virus at the cell membranes. According to the observations of Howatson and Whitmore (1962) and Berkaloff (1963), the cell membranes take part in formation of the outer membrane of the virus particles. This is contrary to our observations on maize mosaic virus, where the membranes of the endoplasmic reticulum do not form part of the virus membranes. Such a conclusion is supported by many electron micrographs, for instance Figs. 3A, 3C, and 5, and also by measurements of particle diameters.

Some years ago it was proposed by Robertson (1959) that the endoplasmic reticulum is open to the cell surface. Recently, Huxley (1964) showed that the transverse system of the sarcoplasmic reticulum of frog muscle opens out directly into the extracellular space. Unpublished investigations in our laboratory indicate that the endoplasmic reticulum in the salivary gland of *P. maidis* indeed communicates with the extracellular duct of the cell.

ACKNOWLEDGMENTS

The authors wish to thank Dr. G. H. Bergold for his support and discussion, Mr. O. Suárez for assistance in the preparation of salivary glands and intestine, and Miss I. Hermanavicius and Miss M. Estevanot for technical help.

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Effect of Particle Length Distribution on Infectivity of Tobacco Mosaic Virus[1]

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The particle length distribution of tobacco mosaic virus purified by four different procedures was determined by electron microscopy. All the samples contained particles shorter than the standard 300 mμ length as well as aggregates of the short and standard lengths. Aggregation was greatest in samples known to contain relatively large amounts of calcium and magnesium ions, particularly when the virus was purified at room temperature. Essentially nonaggregated preparations were made by processing in low concentrations of sodium ethylenediamine tetraacetate.

Some of the preparations mentioned were fractionated by density gradient centrifugation, differential ultracentrifugation, and agar gel column chromatography into samples consisting primarily of short or of standard length units, and their infectivities were compared on *Nicotiana glutinosa*. The fractions containing few or no standard particles proved very low in infectivity, as reported previously by other workers. However those with moderate proportions of short rods were usually more infectious per standard length unit present than were those with low proportions of short particles. The hypothesis is advanced that the enhancement of infectivity found with the shorter rods is a specific effect possibly related to their nucleic acid content and their ability in multiple particle infections to supplement the genetic information carried by the standard particle.

INTRODUCTION

Rod-shaped particles shorter and longer than the predominant 300 mμ length occur in the juice from plants infected with tobacco mosaic virus, TMV, and in the purified virus. The longer particles have been explained by end-to-end aggregation or dimerization of the predominant rods (Lauffer, 1938; Schachman and Williams, 1959). However rod lengths intermediate between 300 and 600 mμ also occur (Lauffer, 1944), showing that aggregation probably also involves combination of shorter and standard lengths. The shorter particles have been ascribed to breakage of the predominant units during the grinding and extraction procedures (Williams and Steere, 1951), but their prevalence in the juice has also suggested (Commoner *et al.*, 1962) that they are present in infected plants. In keeping with the hypothesis that an intact 300 mμ length rod, or its nucleic acid component, is necessary for infection, the shorter particles are generally believed to be noninfectious and the small amount of infectivity sometimes associated with them is explained by the presence of a few standard rods (Sigurgeirsson and Stanley, 1947; Lauffer, 1943).

Recent papers by several authors bear both on the question of the occurrence of the shorter rods in the plant and on their infec-

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tivity. Commoner and co-workers (Commoner and Symington, 1962; Symington *et al.*, 1962) have provided convincing evidence from $C^{14}O_2$ uptake in the infected leaf that the shorter rods do occur and are metabolically active in the plant, and they have concluded that "the product of TMV biosynthesis is a heterogeneous population of ribonucleoprotein rods." A fraction of short rods free from 300 mμ units (as determined by measurement of 583 particles) gave from 0.4 to 2% as many local lesions as a standard preparation, suggesting that the short rods might be slightly infective per se (Commoner *et al.*, 1962). The authors, however, point out that the failure to find standard particles could have been due to a sampling error and the infectivity of the sample explained by the presence of about one 300 mμ length rod per 300 particles. Steere (1963) by treatment of the juice with dilute ethylenediamine tetraacetate (EDTA) to avoid aggregation obtained evidence for the presence of large amounts of the shorter rods in the juice and was successful by agar gel adsorption in obtaining fractions consisting solely of the shorter particles. However, he found no evidence for infectivity in these preparations. Townsley (1963) prepared noninfectious fractions containing only short rods by agar gel electrophoresis.

As shown by experiments in this laboratory, TMV prepared by ultracentrifugation in the absence of buffer salts or after treatment with EDTA forms large amounts of noncompacted components during the third and subsequent cycles in contrast to the virus sedimented in 0.1 *M* phosphate (Loring *et al.*, 1959; Tu, 1960). A number of such fractions examined by Tu contained over 50% by weight of shorter particles but proved comparable in infectivity to the pelleted virus. These results indicated either that the shorter particles may be more infectious under some circumstances than generally accepted or that they may enhance the infectivity of standard particles, possibly in a manner comparable to that of the noninfectious TMV or protein components recently reported (Santilli *et al.*, 1961). In order to study infectivity as a function of standard particle content, TMV was prepared by several procedures and fractionated into samples which by electron micrography contained from about 100% to 5% by weight of particles shorter than 270 mμ with varying amounts in the standard and longer than standard ranges. The infectivity of a fraction was compared either with that of its parent sample or of another fraction of different length distribution prepared at the same time. This paper presents electron micrography characterization of the various preparations and fractions and the results of the activity comparisons.

MATERIALS AND METHODS

Virus preparations. The virus was produced in *Nicotiana tabaccum* var. Turkish grown in a heated greenhouse and harvested near maturity (5–6 weeks after inoculation). The plants were wrapped in paper and stored in a deep-freeze until used. Preparations of different metallic composition were made as follows:

Ca-Mg-TMV. Preparations Ca-Mg-TMV_1, and Ca-Mg-TMV_2 were made by three cycles of differential ultracentrifugation in the cold in the absence of buffer salts as previously described (Loring *et al.*, 1962). The samples dissolved in water[2] were stored at 4° at concentrations of 28.8 A_{260} and 45 A_{260}, respectively, where A_{260} refers to the absorbance with 1 cm light path at 260 mμ in 0.1 *M* potassium phosphate buffer at pH 7. Freshly prepared dialyzed Ca-Mg-TMV gave an absorbance in the range of 3.3–3.5 per milligram per milliliter. Ca-Mg-TMV_3 was prepared in the same manner as Ca-Mg-TMV_1 except that the frozen plants, after thawing, were allowed to stand at room temperature for several days before processing at room temperature.

Na-TMV. Sample Na-TMV_1 was obtained from a first-cycle Ca-Mg-pellet fraction by re-solution in 0.01 *M* Na-EDTA, pH 7.5. The clarified solution was subjected to two additional ultracentrifugal purification cycles in 0.01 *M* Na-EDTA, pH 7.5. The final pellets were redissolved in water and stored in the cold at 39.7 A_{260}. Sample Na-TMV_2 was prepared by making Ca-Mg-TMV_2 0.01 *M* with Na-EDTA at pH 7.5 and allowing

[2] Deionized water was used throughout.

the solution to stand in the cold for several days.

Metal-free TMV. The frozen ground pulp was mixed with one-fifth volume of 0.5 *M* Na-EDTA, pH 8.3, and the juice was extracted by the usual procedures. The virus was subjected to three cycles of differential ultracentrifugation (Loring *et al.*, 1962) with 0.01 *M* Na-EDTA, pH 7.5, used for resolution of the first- and second-cycle pellets. The third-cycle pellets were dissolved in water and dialyzed against 24 liters of water on a rocking-dialyzer (Kunitz and Simms, 1928) over a 3-day period in a cold room.[3] The pH of the final sample dropped to about 6.0. It was stored at an absorbance of 58 A_{260}.

Density gradient fractionation. Sample Ca-Mg-TMV_1 was subjected to density gradient fractionation in 0.01 *M* potassium phosphate-sucrose at pH 7 according to the general procedure of Brakke (1960). The virus was layered at a concentration of 4.5 A_{260} in 0.01 *M* phosphate (3 ml per tube); after centrifugation in a swinging-bucket rotor for 2 hours (54,000 *g*), the solution in each tube was separated into seven equal fractions of 4 ml each. Appreciable amounts of the ultraviolet-absorbing components placed on the gradient were present in each fraction. The uppermost was called Ca-1, the next lower, Ca-2, etc., to Ca-7 for the lowest. The respective fractions from four runs were pooled, and the virus in each was recovered by centrifugation for 2 hours at 30,000 rpm (78,000 *g*). The fourth fraction was brought to an absorbance of 3.7 A_{260} in 0.01 *M* phosphate and subjected to a second density gradient fractionation as before. The respective fractions after virus recovery were labeled Ca-4a, 4b, etc. The main fraction, Ca-4d, gave an absorbance of 3.3 A_{260} and was subjected to a third density gradient centrifugation. The respective recovered fractions were called Ca-4d1, 4d2, etc.

[3] Samples prepared in a comparable manner were found (Eiskamp, 1962) to be essentially free of metallic components: e.g., Na, K, and Ca, 0–5; Mg, Fe, Mn, 0–3 atoms per particle. They gave an absorbance, 260 mμ, of 3.2 ± 0.05 (95% confidence limit) per milligram per milliliter in 0.01 *M* sodium phosphate buffer at pH 7.

Sample Na-TMV_1 was also fractionated in 0.01 *M* phosphate-sucrose at a concentration of 4.5 A_{260}. As with Ca-Mg-TMV_1 seven fractions of 4 ml each were separated from each tube, the respective fractions from two runs were pooled, and the virus was recovered by ultracentrifugation. The fractions were called Na-1 to Na-7, inclusive.

Two additional density gradient fractionations were performed on sample Na-TMV_1, one at 4.5 A_{260}, and one at 1.5 A_{260} in 0.001 *M* potassium phosphate-sucrose. In each case nine fractions were separated per tube with respective volumes from top to bottom of each tube of 4, 4, 2, 2, 2, 2, 2, 4, and 6 ml. Respective fractions from five runs at 4.5 A_{260} and from 8 at 1.5 A_{260} were pooled, and the virus in each was recovered by ultracentrifugation as described. The respective fractions at 4.5 A_{260} were called Na-1a to Na-9a, inclusive, and those at 1.5 A_{260} Na-1b to Na-9b, inclusive.

The distribution of viral components in the respective density gradient fractions from each of the experiments mentioned is shown in Table 1 as the percentage of total absorbance recovered. The main fraction with both types of preparations occurred near the middle, but viral components were distributed throughout the gradient. Larger amounts of ultraviolet-absorbing components were present in the Na-TMV preparations in the layers above the main fraction than with Ca-Mg-TMV. As mentioned in more detail later such fractions contained relatively large amounts of rods shorter than 270 mμ.

Ultracentrifugal fractionation. The metal-free sample was further fractionated by a series of 20-minute ultracentrifuge runs at 24,000 rpm in a no. 30 Beckman-Spinco rotor (50,000 *g*). The virus dissolved in 0.001 *M* Na-EDTA, pH 7.5, at 10 A_{260} (called Na-TMV_3), was centrifuged, and the supernatant liquid was poured off and subjected to a second centrifugation as before. This procedure was repeated for a total of eight times. The pellets were redissolved in 0.001 *M* Na-EDTA, pH 7.5, giving fractions, Na-P1, P2, P3, P4, P5, P6, P7, and P8. Portions of the supernatant fractions from the first centrifugation, Na-S1 and the last,

TABLE 1

VIRUS DISTRIBUTION AS PERCENTAGE OF TOTAL A_{260} RECOVERED FROM DIFFERENT FRACTIONS AFTER DENSITY GRADIENT CENTRIFUGATION

Virus sample	Fraction[a]								
	1	2	3	4	5	6	7	8	9
Ca-Mg-TMV_1[b]	0.3	2	22	58	14	1	1	—	—
Ca-4[b]	0.2	0.4	10	67	16	4	2	—	—
Ca-4d[b]	0.3	0.5	18	65	11	3	2	—	—
Na-TMV_1[b]	0.2	1	28	55	9	4	2	—	—
Na-TMV_1, 4.5 A_{260}[c]	<1	1	8	33	31	12	5	5	5
Na-TMV_1, 1.5 A_{260}[c]	<1	2	8	20	35	18	7	6	6

[a] All fractions are numbered in order of removal from top of centrifuge tube.

[b] Centrifuged in 0.01 M phosphate-sucrose; fractions of 4 ml each were removed and pooled as described in text.

[c] Centrifuged in 0.001 M phosphate-sucrose; fractions of 4, 4, 2, 2, 2, 2, 2, 4, and 6 ml were removed and pooled as described in text.

Na-S8, were also saved. The first pellet, Na-P1, was redissolved in 0.001 M Na-EDTA, pH 7.5, brought to about the same concentration as that used initially, and subjected to another centrifugation at 24,000 rpm in the no. 30 rotor, giving pellet fraction Na-P1a and supernatant fraction Na-S1a. The P1a fraction was subjected to another ultracentrifugal fractionation as before, giving pellet fraction Na-P1b and supernatant fraction Na-S1b.

Agar gel column fractionation. Several fractions containing different proportions of short, standard, and long particles were made from the metal-free sample by agar gel column fractionations (Steere and Ackers, 1962a,b).

A column 1.25 cm in diameter and 125 cm long was filled with 1% agar gel particles, made by grinding the gel prepared in water in a Waring Blendor, and using those particles which could be forced through a 40-mesh but were retained by a 60-mesh screen. The column was equilibrated with 0.01 M Na-EDTA, pH 7.5, by washing with this solution until the effluent absorbances at 260 and 320 mμ were approximately equal to those of the solvent. Two milliliters of the metal-free virus ($A_{260} = 50$) were layered on the agar and washed slowly (about 2 ml/hr) through the column with 0.01 M EDTA. Fractions were collected every hour. Total A_{260} in the effluent was 97% of that applied, indicating that nearly all the virus passed through the column. G1 is an early, and G2 is a late, fraction from this run.

A second column was filled with 2% agar particles and equilibrated with 0.001 M Na-EDTA, pH 7.6. Two milliliters of the metal-free virus were filtered through this column. G3 and G4 are early fractions and G5 is a late fraction from this run.

Electron microscopy. Electron micrographs were made of most of the virus samples mentioned and of their respective fractions by standard techniques (Williams, 1954; also personal communications). The virus was diluted to approximately 0.1 absorbance at 260 mμ in 0.05% bovine serum albumin. The resulting solution was sprayed from a Vaponefrin atomizer (Vaponefrin Co., Portland, Oregon) onto a stainless steel electron microscope grid previously covered with a collodion film. The sprayed grids were air dried, shadowed with chromium, and covered with a carbon film in a Kinney SC-3 vacuum evaporator. Single droplets on the grids were photographed at a magnification of 5000 × in an Hitachi model 10 electron microscope. Four times enlarged prints were made of the resulting negatives, and the lengths of individual particles were measured with a ruler and a magnifying glass, usually to the nearest 1/60 inch, (or including the magnification factor to 20 mμ). The number of particles of each length was counted and converted to relative weight by multiplying by the length. The sum of the relative

weights of particles shorter than 270 mμ, of those between 270 and 330 mμ, and of those longer than 330 mμ was determined, and the weight percentage of each of these three classes was calculated. The accuracy with which the relative number of particles measured in any one category represents the true value in the sample depends on the total number of all particles measured, n. It can be approximated from the standard deviation, σ, where $\sigma = \sqrt{pqn}$ and p represents the probability of a particle being in a given category (close to n_1/n where n_1 is the number of particles measured in that category) and q, the probability of not being in the category (Bowker and Lieberman, 1959). The value of σ is maximal when $p = \frac{1}{2}$ and for $n = 1000$ is 15.8 or for $n = 100$ is 5. The respective values of 2 σ (corresponding to 95% confidence limits) are ± 31.6 and ± 10 or about ± 3% and ± 10% of the total number of particles measured in each case. As the value of σ is usually less than the maximum, the actual 95% confidence limits for number percentage would be somewhat less than ± 3% and ± 10% of the number of particles measured. The corresponding confidence limits in weight percent would be somewhat different but of the same orders of magnitude.

Infectivity tests. The infectivity of a fraction was determined from the number of local lesions produced when it was inoculated onto half-leaves of *Nicotiana glutinosa* L (Loring, 1937) in comparison with either the parent sample or with a control of known length distribution. The concentrated virus was diluted with cold 0.1 *M* potassium phosphate buffer at pH 7.5 to concentrations expected to give between 10 and 100 lesions per half-leaf. In most of the tests both the fraction and the original were diluted to the same concentrations for inoculation, ordinarily 0.01 or 0.02 A_{260} for tests conducted during the spring and summer months, and 0.1 A_{260} during fall and winter. When inoculations were made on leaves previously dusted with 500-mesh Carborundum to increase lesion count, virus at 0.0005 to 0.001 A_{260} was used. At least two plants (approximately 6 leaves per plant), and usually four, were used for each test. The leaves were picked after lesions had developed (usually 5–7 days) and the lesions were counted. The ratio of the number of lesions produced by the fraction to that produced by an equal concentration of the control was called the relative infectivity of the fraction.

Several authors (Loring, 1937; Spencer and Price, 1943) have indicated that differences in concentration of infectious material of the order of ± 20% can be detected from the lesion ratio but that the number of lesions is not a linear function of slope one of the concentration. Tests in our laboratory have confirmed both conclusions.[4] As a result it is desirable to keep the relative infectivity as near unity as possible. In order to do this, fractions containing few standard particles were inoculated at such concentrations as to provide as nearly as possible the same number of standard particles per unit volume, and thus theoretically the same number of lesions as the control. In such cases relative infectivity was obtained by dividing the lesion ratio (fraction to control) by the ratio of the respective concentrations.

RESULTS

The frequency distribution diagrams of four of the virus preparatons are reproduced in Fig. 1. The weight percentages of the three classes of rods present (namely those shorter than 270 mμ, those between 270 and 330 mμ, and those longer than 330 mμ) calculated for Ca-Mg-TMV_1, Ca-Mg-TMV_2, Ca-Mg-TMV_3, Na-TMV_1, Na-TMV_2, and Na-TMV_3 and the number of particles measured in each case are summarized in Table 2. It is apparent that the length distribution of TMV varied widely depending on the preparative procedure used for virus purification. Virus prepared in the cold in the absence of buffer salts is known to contain relatively large amounts of Ca^{++} and Mg^{++} (Loring *et al.*, 1962) and, as shown in

[4] The differences in lesion counts in most of the infectivity tests were examined for significance by the t test for paired variates (Bowker and Lieberman, 1959). The probability of significance was generally 80% or greater for 20% differences in lesion counts and 95% or greater for 30% differences.

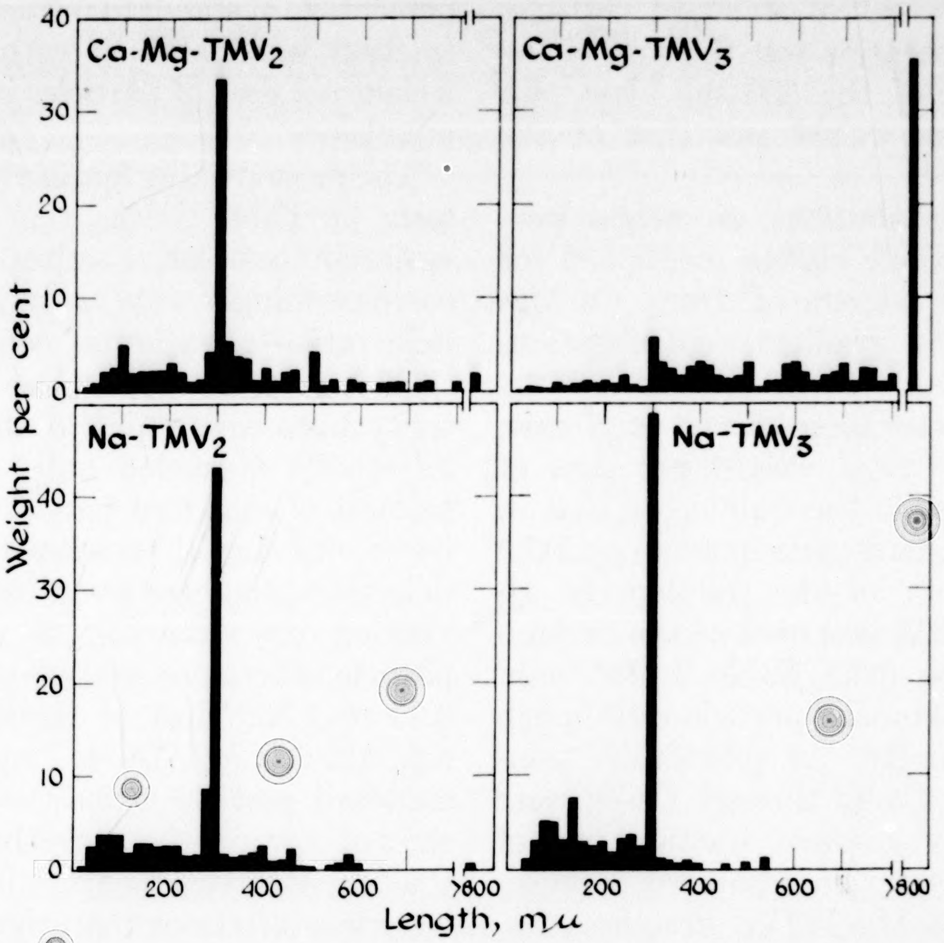

FIG. 1. Dependence of rod length distribution of TMV on preparative procedure.

TABLE 2

ROD LENGTH DISTRIBUTION OF TMV PREPARATIONS

Preparation	Weight percentages			Number of rods measured
	<270 mμ	270–330 mμ	>330 mμ	
Ca-Mg-TMV$_1$	17	52	31	159
Ca-Mg-TMV$_2$	27	44	29	411
Ca-Mg-TMV$_3$	6	13	81	333
Na-TMV$_1$	33	52	15	196
Na-TMV$_2$	32	55	13	645
Na-TMV$_3$	43	52	5	457

Fig. 1 for Ca-Mg-TMV$_2$, contained appreciable amounts (about 30% by weight) of rods longer than 330 mμ. The effect of prolonged exposure to room temperature during processing on the state of aggregation is shown by the distribution diagram for Ca-Mg-TMV$_3$. About 80 weight per cent of the virus in this sample was present as particles longer than 330 mμ with about 35% longer than 800 mμ. When Ca-Mg-TMV$_2$ was treated with 0.01 *M* EDTA at pH 7.5 and divalent metal ions were largely sequestered, the weight percentage of longer rods in the resulting virus, called Na-TMV$_2$, decreased to 13%. The metal-free virus in the presence of 0.001 *M* Na-EDTA at pH 7.5 (Na-TMV$_3$) was almost free of aggregated particles but contained the largest amount (about 45% by weight) of rods shorter than 270 mμ. As Ca-Mg-TMV$_3$ and Na-TMV$_3$ produced about the same number of lesions when inoculated at the same absorbance in 0.1 *M* potassium phosphate at pH 7, it is apparent that infectivity was not greatly altered by the end-to-end aggregation of the standard length rods. Electron micrographs of Ca-Mg-TMV$_3$ in 0.1 *M* phosphate showed little dissociation, and it appears probable that rods longer than 330 mμ caused infection at least as well as standard length rods. The longer rods in Ca-Mg-TMV$_3$ were dissociated significantly but not completely at pH 8.5 in 0.01 *M* Na-EDTA, forming about two-thirds by weight of rods in the 300 mμ class and about one-third in the shorter category. In attempting to relate infectivity to the weight percentage of 300 mμ particles present, the assumption was accordingly made that two-thirds of the longer particles consisted of the standard units and that the

total weight per cent of standard particles present in a preparation was the sum of the weight per cent of the 300 mμ class plus two-thirds of the weight per cent of the longer rods.

The length distributions, as weight percentages of the three classes mentioned for several fractions separated from Ca-Mg-TMV$_1$ by density gradient centrifugation, are shown in Table 3. Also listed are the number of particles measured in each case, the ratio of the total weight per cent of standard particles in the fraction to that in the control (standard particle ratio) and the relative infectivity of the fraction. In all cases Ca-Mg-TMV$_1$ was used as the control.

It can be seen from Table 3 that only Ca-2 showed a standard particle ratio much different from unity. As previously mentioned, fractions Ca-4d through Ca-4g were made by density gradient fractionation of Ca-4, the main fraction separated from an initial run on Ca-Mg-TMV$_1$. Fractions Ca-4d3 through Ca-4d7 resulted from an additional run on Ca-4d. This sequential density gradient centrifugation was performed in an attempt to provide fractions with a higher percentage of standard particles. However no fraction was obtained with more than 70 weight per cent of particles in the 270 to 330 mμ class.

The most striking feature of the infectivity tests in Table 3 was the extremely wide variation in relative infectivity without a correspondingly wide range in standard particle ratio—a variation considerably wider than expected on the basis that the infectivity tests were valid to about ± 20%. If infectivity depended only on the relative amount of standard particles, then relative infectivity should be equal to standard particle ratio. In these tests, however, fractions varying only from 0.95 to 1.17 in standard particle ratio gave relative infectivities from 0.35 to 1.35. Most of the lowest, fractions, e.g., Ca-6, Ca-7, Ca-4g, and Ca-4d7, gave standard particle ratios close to unity, but showed low relative infectivities, indicating that the standard particles in these fractions were less infectious than those in the parent samples. Also of significance in Table 3 was the very low relative infectivity of Ca-2, a fraction consisting almost entirely of short particles. This is in accord with the previ-

TABLE 3

WEIGHT PERCENTAGES IN <270 mμ, 270–330 mμ, AND >330 mμ LENGTH CLASSES, NUMBER OF PARTICLES COUNTED, STANDARD PARTICLE RATIOS, AND RELATIVE INFECTIVITIES OF FRACTIONS SEPARATED FROM Ca-Mg-TMV$_1$ BY DENSITY GRADIENT CENTRIFUGATION

Fraction	Weight percentages			Number of particles	Standard particle ratio	Relative infectivity[a]
	<270 mμ	270–330 mμ	>330 mμ			
Ca-2	>99	<1	<1	1253	0.00	<0.01
Ca-3	17	47	44	183	1.06	0.61
Ca-4	10	65	25	363	1.12	0.97
Ca-5	7	41	52	225	1.02	0.88
Ca-6	11	55	34	125	1.07	0.35
Ca-7	25	58	17	35	0.95	0.35
Ca-4d	8	69	22	164	1.15	1.00
Ca-4e	9	54	37	188	1.08	1.35
Ca-4g	18	56	26	53	1.01	0.69
Ca-4d3	26	67	7	182	0.99	1.18
Ca-4d4	5	64	31	118	1.17	1.11
Ca-4d6	12	56	32	154	1.06	1.04
Ca-4d7	11	57	32	76	1.08	0.40

[a] Ca-2 was inoculated without Carborundum at 0.1 A_{260}, as compared to 0.01 A_{260} for the control, and gave a lesion ratio of 0.06. Ca-3 to Ca-4d, inclusive, and their controls were inoculated at 0.0005 A by rubbing with a gauze pad after an area 1–2 inches in diameter near the petiole of each leaf had been sprinkled lightly with 500-mesh Carborundum. The remaining fractions and their controls were inoculated without Carborundum at 0.01 A_{260}.

TABLE 4

WEIGHT PERCENTAGES IN <270 mμ, 270–330 mμ, AND >330 mμ LENGTH CLASSES, NUMBERS OF PARTICLES COUNTED, STANDARD PARTICLE RATIOS AND RELATIVE INFECTIVITIES OF FRACTIONS SEPARATED FROM Na-TMV$_1$ BY DENSITY GRADIENT CENTRIFUGATION

Fraction	Weight percentages			Number of particles	Standard particle ratio	Relative infectivity[a]
	<270 mμ	270–330 mμ	>330 mμ			
Na-3	53	46	0	143	0.74	0.72
Na-4	14	69	17	126	1.30	1.4[illegible]
Na-5	20	51	28	121	1.12	1.11
Na-7	30	68	3	69	1.13	1.04
Na-2a	100	0	0	226	0.00	<0.01
Na-3a	71	26	3	735	0.45	0.69
Na-3a	71	26	3	735	0.39	0.55
Na-4a	23	60	18	424	1.16	1.00
Na-5a	10	60	30	138	1.29	0.94
Na-9a	20	61	19	91	1.19	0.45
Na-2b	100	0	0	155	0.00	<0.0003
Na-3b	58	34	8	419	0.54	0.56
Na-4b	25	62	13	148	1.14	0.78
Na-5b	21	70	9	80	1.22	0.67
Na-9b	19	58	23	41	1.15	0.35

[a] Na-TMV$_1$ was used as the control for infectivity tests except for the second test on Na-3a, where Na-4a was used. Fractions Na-3, Na-4, Na-5, and Na-7 were prepared in 0.01 *M* phosphate; other fractions were prepared in 0.001 *M* phosphate. All fractions and controls were inoculated at 0.02 A_{260} without Carborundum with exceptions as follows: Na-2a was inoculated at 0.14 versus control at 0.02 and gave a lesion ratio of 0.05; Na-3a, second test, was inoculated at 0.039 versus control at 0.015; and Na-2b was inoculated at 0.094 versus control at 0.001 and gave a lesion ratio of 0.03.

ously quoted results of other investigators on fractions consisting essentially of the short rods.

The length distributions and infectivities of the fractions separated by density gradient centrifugation of Na-TMV$_1$ are shown in Table 4. The uppermost fractions, e.g., Na-2a and Na-2b, contained few or no standard particles and gave relative infectivities less than 0.01. In other upper fractions, e.g., Na-3, Na-3a, and Na-3b, the samples contained a majority of the shorter rods and gave relative infectivities close to or higher than those expected from their standard particle ratios. Middle or lower fractions were either comparable in length distribution to the virus placed on the gradient or contained somewhat greater amounts of the standard rods. Relative infectivity of fractions separated in 0.01 *M* phosphate gave relatively good agreement with standard particle ratios. Most of those, however, in which 0.001 *M* phosphate was used (and particularly the lowest fraction in the gradient, i.e., Na-9a and Na-9b) were low in infectivity in comparison to the number of standard particles present.

The length distributions and infectivities of fractions separated from Na-TMV$_3$ by differential centrifugation and by agar gel column fractionation are given in Table 5. It can be seen that both procedures also gave fractions varying widely in standard particle ratios (0.25–1.64). Those fractions with standard particle ratios greater than 1 gave higher relative infectivities than those with ratios less than 1. However inspection of the data for fractions consisting primarily of short particles (i.e., Na-S6, Na-S8, G2, and G5) indicated that most of these had a higher relative infectivity than standard particle ratio; conversely most fractions with relatively high standard particle ratios (i.e., 1.2 or greater) had significantly lower relative infectivities than standard particle ratios.

If relative infectivity is equal to standard particle ratio, it is apparent that relative

TABLE 5

WEIGHT PERCENTAGES IN <270 mμ, 270–330 mμ, AND >330 mμ LENGTH CLASSES, NUMBERS OF PARTICLES COUNTED, STANDARD PARTICLE RATIOS, AND RELATIVE INFECTIVITIES OF FRACTIONS SEPARATED FROM Na-TMV_3 BY DIFFERENTIAL CENTRIFUGATION (Na SERIES) AND AGAR GEL COLUMN CHROMATOGRAPHY (G SERIES)

Fraction	Weight percentages			Number of particles	Standard particle ratio	Relative infectivity[a]
	<270 mμ	270–330 mμ	>330 mμ			
Na-P1	18	63	19	377	1.37	0.83
Na-S1	41	52	6	189	1.01	1.12
Na-P1a	14	75	11	284	1.49	1.31
Na-S1a	20	67	13	228	1.37	0.81
Na-P1b	16	67	17	139	1.41	0.83
Na-S1b	19	68	13	440	1.38	1.14
Na-P2	25	66	9	1033	1.30	1.16
Na-P3	26	67	7	203	1.29	0.75
Na-P4	29	64	7	502	1.24	1.03
Na-P5	32	58	10	601	1.17	0.99
Na-P6	42	53	4	217	1.00	0.84
Na-S6	74	26	1	587	0.48	0.67
Na-S8	79	19	2	1666	0.37	0.49
Na-S8	79	19	2	1666	0.25	0.23
G1	6	84	10	239	1.64	0.75
G2	69	27	3	223	0.32	0.46
G3	14	74	12	350	1.48	1.18
G4	15	68	17	361	1.43	0.98
G5	70	29	1	604	0.53	0.69

[a] Na-TMV_3 was used as the control for infectivity tests except for the second test on Na-S8, where Na-P1a was used, and for the test on G2, where G1 was used. Fractions Na-P1 to Na-S6, inclusive, and their controls were inoculated at 0.02 A_{260} ; Na-S8 was inoculated at 0.054 and 0.08 versus 0.02 for the controls; G1 at 0.0133 versus 0.02; G2 at 0.09 versus 0.03; G_3 and G4 and their controls at 0.1; and G5 at 0.13 versus 0.1.

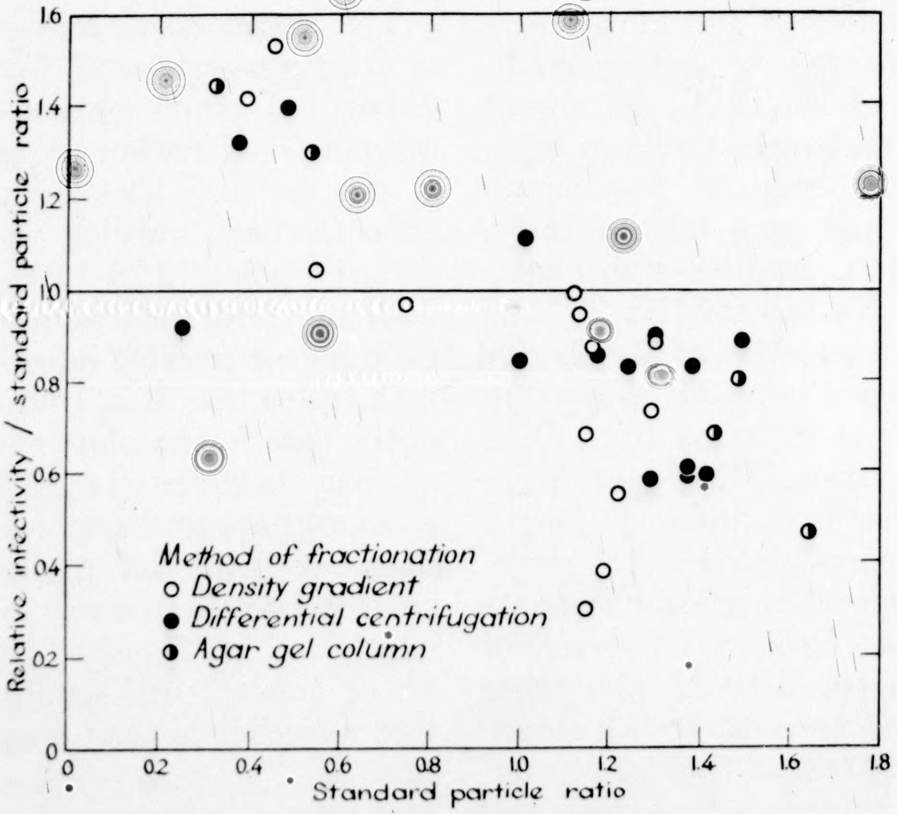

FIG. 2. Relative infectivity: standard particle ratio of Na-TMV fractions as a function of standard particle ratio.

infectivity divided by standard particle ratio for any fraction should always be 1 regardless of the numerical value of the standard particle ratio. This quotient, which represents relative infectivity per standard particle, was calculated for each of the fractions listed in Tables 4 and 5 and plotted in Fig. 2 against standard particle ratio.[5] It is clear that the points do not follow a straight line of zero slope at an ordinate of 1 as shown in the figure; fractions with low standard particle ratios were generally above, and those with high standard particle ratios generally below the unity line.

The data presented for Na-TMV in Fig. 2 as mentioned, were based on the assumption that infectivity was due both to the weight of the 300 mμ and to two-thirds of the weight of the longer particles. It was also of interest to examine the data based on the two limiting assumptions that the longer particles were either noninfectious, or that they were as infectious, on a weight basis, as the standard particles. Graphs of relative infectivity: standard particle ratio versus standard particle ratio on each of these bases showed that in both cases the infectivity of fractions with low relative amounts of standard particles was higher, and that of fractions with high relative amounts of standard particles lower, than would be predicted on the basis of strict proportionality of infectivity to standard particle content.

DISCUSSION

The data on particle length distribution of the four types of TMV preparations (Fig. 1) studied in this report are in accord with the recent conclusions of several authors (Symington *et al.*, 1962; Steere, 1963; Townsley, 1963) that shorter than standard length particles are present in the infected plant. The data have also clarified some of the factors concerned with the occurrence of the shorter rods and with their aggregation with the standard length units into rods of even longer lengths. The extent of such aggregation was dependent both on the temperature employed during purification and on the presence of divalent cations. After three cycles of differential centrifugation in water at temperatures near zero, e.g., Ca-Mg-TMV_1, and Ca-Mg-TMV_2, the major viral component was the standard 300 mμ length unit, but large amounts, ca. 20–30% each by weight of both shorter and longer rods, were also present. The aggregates consisted not only of dimers and trimers of standard length units, but included components of almost all possible intermediate lengths as well. Ca-Mg-TMV samples processed at room temperature, however, e.g., Ca-Mg-TMV_3 contained only small amounts of the shorter and standard rods, but the aggregated particles increased to about 80% by weight.[6] As shown in Fig. 1, about 45% of the virus was distributed almost uniformly in lengths from 300 to 800 mμ with about 35% in lengths of the order of trimers of the standard length. That the presence of divalent metals influences the extent of such aggregation reactions was indicated by the length distribution of Ca-Mg-TMV_1 and Ca-Mg-TMV_2 after treatment with Na-EDTA. Striking decreases occurred in the weight percentages of rods longer than 330 mμ with corresponding increases in the content of shorter rods. Dissociation also occurred with the more highly aggregated sample after EDTA treatment, but more of the virus remained aggregated, indicating that the aggregation reactions taking place at room temperature may be only partially reversible. The results, in general, confirm previous reports from this and other laboratories (Loring *et al.*, 1959; Tu, 1960; Symington *et al.*, 1962; Steere, 1963) that relatively large amounts of the shorter rods may occur in the purified preparations, which have been freed or relatively freed from divalent cations.[7]

[5] The data of Table 3 were not included in this figure because little apparent fractionation occurred when Ca-Mg-TMV was subjected to density gradient centrifugation. It can be readily seen that their inclusion would have little effect on the general appearance of the plot.

[6] It should be noted that even higher temperatures, ca. 50° for 5 minutes, have been used to prepare virus with only 2–6% short rods (Williams and Steere, 1951). It is probable that here too aggregation of short and standard length units was high, since calculations based on the published data show that 25–40% by weight of the virus was present in the form of rods of longer than standard length.

[7] While no metal analyses were reported by Symington *et al.* (1962) for the preparations sub-

The infectivity data on fractions of known length distribution confirm previously quoted conclusions of many authors that the infectivity of a preparation is greatly dependent on the presence of particles of about 300 mμ length. It is apparent, however, from the length distribution and relative infectivities of the lowest density gradient fractions, e.g., Ca-6, Ca-7, Ca-4g, Ca-4d7, Na-9a, Na-5b, and Na-9b, in Tables 3 and 4 that fractions having amounts of standard length units comparable to their controls were often low in infectivity. It seems evident, therefore, that rods of 300 mμ length vary in their abilities to produce lesions in *N. glutinosa* and must be considered inhomogeneous from this standpoint. Data of Commoner *et al.* (1962, Table 1) for samples isolated at various times after inoculation and having comparable standard particle contents also support this conclusion.

The infectivity data on fractions consisting primarily of the short rods confirm previously quoted conclusions of a number of authors that such fractions are either noninfectious or poorly so. Sufficiently precise data, however, were not obtained to establish with certainty whether the small amounts of activity associated with such fractions were due to small particles per se or to a few standard length units still present. Removal of the shorter rods in general failed to increase the infectivity of a fraction above that found in the parent preparation and in many instances resulted in decreases in infectivity. Such a result was particularly striking with Na-P1, Na-S1a, Na-P1b, Na-P3, and G1 (Tables 4 and 5), which gave standard particle ratios in the range 1.29 to 1.64 as compared to their controls but in all instances were significantly less infectious. These results are in agreement with those mentioned earlier that standard length units of low infectivity occur in the purified preparations and were in fact concentrated in the last named fractions.

In contrast to the decrease in infectivity of the fractions just mentioned, increases in the relative amounts of the shorter rods enhanced the infectivities of the standard units present in Na3a, Na-S6, Na-S8, G2, and G5. These results are, therefore, in general agreement with previous reports from this laboratory (Loring *et al.*, 1959; Tu, 1960) that preparations containing large amounts of short particles may be relatively high in infectivity.

The effect of the shorter rods may be nonspecific like the reported effect of bovine serum albumin or reconstituted noninfectious TMV on virus infectivity (Santilli *et al.*, 1961). These workers reported that in virus solutions at 0.5 μg/ml these substances enhance infectivity by about 30–40% when present at concentrations of the order of 10 μg/ml but reduce infectivity drastically at higher concentrations. These conclusions were confirmed in this laboratory for bovine serum albumin for inoculations made in the presence of Carborundum at virus concentrations comparable to those used by these workers. At the virus concentration employed in many of the present tests (33 μg/ml in 0.1 *M* phosphate at pH 7.5 without Carborundum), however, bovine serum albumin over a concentration range of from 2 to 150 μg/ml consistently reduced infectivity about 20%. It seems possible, therefore, that the enhancement of infectivity in the presence of the shorter particles may be a specific effect related to the transfer of the coding information necessary for infectivity.

In order that such a theory be valid, it must be assumed that while infection by single particles is possible the probability of infection is increased by the entrance of more than one particle into the damaged cell. Preliminary evidence for this assumption has been obtained and will be presented in detail in a future publication. A series of tests was conducted in which the infectivities of fractions containing high percentages of short particles were compared with those of others containing high percentages of standard particles, both at low virus concentrations (using Carborundum to increase the number of susceptible cells) and at high virus concentrations. The results indicate that infectivity enhancement by small particles may be limited to tests made at the higher concentrations in the absence of Car-

jected to repeated isoelectric precipitation, this type of treatment should also decrease the divalent metal content of the purified virus.

borundum where the probability is higher that more than one particle could enter the susceptible cell.

A second necessary assumption is that the approximately 6400 nucleotide units present in the standard length particle carry coding information for proteins other than the coat protein, probably enzymes required for virus biosynthesis.[8] The TMV coat protein is known to contain 158 amino acid residues (Tsugita *et al.*, 1960) and on the basis of the currently accepted triplet code (Crick *et al.*, 1961) would require less than one-thirteenth of the total number of nucleotide units of the standard virus rod. Direct evidence that this is the case has recently been presented in a study of 117 TMV mutants arising from deamination of the RNA bases by nitrous acid (Wittmann, 1962). Eighty-two of the 117 mutants found showed differences in symptoms from the original strain, but had the same amino acid composition, and thus almost certainly the same protein coat. Wittmann concluded from a comparison of the number of deaminations per mutation that probably less than 500 of the 6400 nucleotides were concerned with coat-protein coding. Accordingly most of the nucleotide units of the virus may be concerned with coding for other proteins required for virus biosynthesis. If this is the case, the RNA's from the shorter viral units could serve as templates for some of these, supplementing the information carried by the standard particles and thus increasing their infectivity. The failure of the fraction consisting solely of shorter rods to cause infection is explained if the synthesis of a TMV rod occurs linearly (Commoner *et al.*, 1959) and always begins at the same end of the RNA chain. The shorter rods, as particles interrupted at various stages of production, would not contain the complete sequence of genetic information and so would not be infectious by themselves.

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[8] Coding for such enzymes has been demonstrated for the bacterial viruses including T_2 and T_4 (Mahler and Fraser, 1961).

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Rescue of One Phenotype in Mixed Infections with Heat-Defective Mutants of Type 1 Poliovirus

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When cells are mixedly infected with two heat-defective (*hd*) poliovirus mutants at temperatures above those that are optimal for growth of these mutants, the virus yield is 4–14 times the sum of the yields of each mutant when grown separately under identical conditions ("leak" yields). The mixed infection yields are only one thousandth of the yield of wild type (hd^+) under these conditions. The leak and mixed infection yields are 90–99% mutant rather than hd^+, but only one of the mutants was detected in the mixed infection yields. Any rescue of the *hd* mutants by hd^+ was too small to be detected.

INTRODUCTION

Conditional lethal mutations of bacteriophage T4, which prevent its growth either in certain hosts or at higher temperatures, have been used with great effect (Epstein *et al.*, 1963) to map the T4 genome and to indicate many of its gene functions. The discovery that 5-fluorouracil produces many heat defective (*hd*) mutants in poliovirus (Cooper, 1964) suggests the possibility of a similar approach to the genetic analysis of a small animal ribovirus.

The *hd* mutants that have been isolated are now being examined in several ways. One way is to classify them by means of a complementation test, with the object of defining those groups of mutants which have defects in the same gene. This test showed that mixed infection at high temperature with many but not all pairs of *hd* mutants gave an enhanced yield. The enhancement was always very small, however.

This paper describes the complementation test and reports the results of some experiments in which the interaction between two of the mutants is examined in some detail with the object of defining optimal conditions. It is shown that, for this particular pair at least, the enhancement was not reciprocal within the sensitivity of the test.

METHODS

Temperature control. Well-circulated water baths controlled by Braun (Melsungen, Germany) Thermomix II mercury contact units maintained temperature to ±0.05°; the most convenient monitors were clinical thermometers graduated in 0.1°C. Tubes were stoppered and were immersed to a depth of 3 cm in open racks; maximum temperature was reached within 3 minutes. Plaque assays of virus at high temperatures were made with a bicarbonate medium (Cooper, 1961) contained in screw-capped bottles immersed inverted in water baths in open baskets, in such a way as not to impede water circulation. In some experiments (indicated in the text) petri plates were enclosed in a sealed box that was immersed in a water bath at a supraoptimal temperature, but this method was discontinued because poor initial temperature control produced a spuriously high plaque count with the *hd* mutants.

Infectivity assays. The agar cell-suspension plaque method (Cooper, 1961) was used, with the human amnion strain U cells

FIG. 1. Osmiophilic particles in the salivary gland of the leafhopper *Endria inimica* Say. Particles in cross and longitudinal orientations.

Aphid Honeydew as Inoculum for the Injection of Pea Aphids with Pea-Enation Mosaic Virus[1]

Studies on the persistent aphid-borne viruses are limited by the lack of simple bioassays. Since most of these viruses are not juice inoculable, vectors are required for the bioassay of various virus-containing extracts. Carter (*3*) demonstrated that the leafhopper *Circulifer tenellus* (Baker) transmitted curly top virus after feeding through a membrane into solutions containing virus. Storey (*17*) repeated this technique with maize streak virus and in addition showed that leafhoppers transmitted virus after being injected with infectious plant extracts (*16*). The needle injection technique has become rather commonly used for leafhopper-borne viruses.

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These two bioassay techniques have been recently applied to the aphid-borne persistent viruses. Barley yellow dwarf virus has been transmitted by the English grain aphid, *Macrosiphum granarium* (Kirby), and by the apple grain aphid *Rhopalosiphum padi* (L.) after feeding on liquid extracts from diseased plants (*12, 13*). Although this is the only published example of successful acquisition of a persistent aphid-borne virus by the artificial feeding technique, perhaps the recent successes of Mittler and Dadd (*9*) and of Auclair and Cartier (*2*) in the artificial rearing of aphids will encourage further trials.

More success has been obtained with needle inoculation of aphids, since four viruses have been transmitted by this method. Transmission of potato leaf roll virus has been reported after injection of green peach aphids, *Myzus persicae* (Sulzer),

with infectious hemolymph or extracts from viruliferous aphids (*4, 5, 7, 15*). Limited success with beet yellow-net virus was reported by Harrison (*5*). Although Heinze (*6*) failed to obtain transmission of pea-enation mosaic virus by injection of hemolymph from viruliferous pea aphids, *Acyrthosiphon pisum* (Harris), later work by Schmidt (*14*) and by Nault *et al.* (*11*) showed that limited transmission could be obtained. Mueller and Rochow (*10*) reported transmission of barley yellow dwarf virus by both the English grain aphid and the apple grain aphid after injection of inoculum from plant or aphid extracts or with hemolymph from viruliferous aphids.

Certain studies on vector-virus relationships, e.g., the relationship between latent period and/or retention to virus dosage, can only be done by a bioassay technique such as artificial feeding or injection. Recently we have spent considerable time investigating some of these vector-virus relationships of the pea aphid and pea-enation mosaic virus (*18*); in order to utilize the injection technique in further studies, we have searched for a suitable source of inoculum.

The plants for the experiments were sweetpea, *Lathyrus odoratus* L., raised from seed in individual 3-inch plastic pots filled with pasteurized soil. The test plants were generally used about 7 days after planting, and virus source plants were used within 2 weeks after the appearance of symptoms. The virus was a strain of pea-enation mosaic virus isolated from a sweetpea plant growing in a garden in Berkeley, California. The virus originally was kept in the greenhouse in bur clover, *Medicago hispida* Gaertn., but for the last two years it has been subinoculated into sweetpea. Nymphs and apterae from a clonal line of the pea aphid vector were raised in colonies on sweetpea, and the preliminary experimental work reported here was done in a 6-month period beginning in May, 1964.

The aphids injected were mainly late instar nymphs, but some adults were also used. Aphids were anesthetized with moistened CO_2, placed on their sides on a piece of rough cardboard on the stage of a stereomicroscope, and injected laterally near the cornicle by means of a twice-pulled glass needle.[2] The injection needle was connected to an air supply by rubber tubing and a glass T-joint; closure of one end of the T-joint forced inoculum out of the needle into the aphid.

Plant extracts were prepared by grinding an equal (w/v) amount of diseased tissue and solvent containing 0.05 *M* K_2HPO_4 and 0.01 *M* Na_2SO_3 in a mortar and sqeezing it through two thicknesses of cheesecloth. The fluid was then centrifuged at 1600 *g* for 10 minutes, and the supernatant was used for the injections.

The plant extracts were drawn up into the needle by connecting it to a small vacuum pump.[3] The amount of liquid drawn into the needle was sufficient to inject two or three dozen aphids.

In the hemolymph tests, the donor aphids were anesthetized and placed on rough cardboard on the stage of the stereomicroscope. A leg was cut off, and the hemolymph that oozed from the wound was collected in an injection needle by capillary action. This material was immediately injected into a recipient aphid.

In the honeydew tests, the plants on which the donor aphids were feeding were caged with glass vials. Small droplets of honeydew ejected to the sides of the vials by the aphids were picked up in the needles by capillary action. One droplet from an adult donor aphid was sufficient to inject an average of 5 aphids. If the honeydew collections were to be pooled, successive droplets were collected in a finely drawn glass tube and then mixed by blowing the liquid out and collecting it again in the tube. The injection needles were then filled from this pooled honeydew.

Injected aphids in all experiments were caged on healthy sweetpea test plants in cellulose nitrate cages, and the insects were serially transferred to additional test plants at varying intervals for several days. The test plants were kept in a Sherer-Gillett model CEL 512-317 plant growth chamber

[2] The injection needles were pulled automatically with a device designed and constructed by Professor R. Craig, Division of Entomology and Acarology, University of California, Berkeley.

[3] Vacuum Tweezer System, model V-100, Ultrasonic Laboratories, Inc., 1780 St. Georges Avenue, Rahway, New Jersey.

during the inoculation access period. The chamber was programmed to have a temperature of 20°C with continuous light. At the end of the inoculation access period, the aphids were removed from the plants, and the plants were fumigated with nicotine before being placed in the greenhouse to complete the incubation period.

A low rate of transmission was obtained with aphids injected with crude plant extracts. Of 49 aphids injected and tested singly, only 3 transmitted virus.

Only eight of 72 recipient aphids transmitted virus after each had been injected with hemolymph from a viruliferous donor aphid. With pooled hemolymph, collected in a single needle from the wounds of several aphids that had had access to a virus-infected plant, 5 of 60 injected aphids transmitted virus.

A high rate of transmission was achieved by injecting honeydew excreted by aphids feeding on virus-infected plants. In the initial trials, 24 of 50 test plants became infected after each had been fed upon by 2 injected aphids. This corresponds to an expected transmission rate of approximately 28 per cent with single insects. Groups of 4 and 5 injected aphids per plant transmitted to 18 of 20 test plants, a rate of transmission too high to be reliably expressed in terms of single-insect expectancy.

To test how soon infectious honeydew would be excreted, a group of aphids was transferred from healthy plants to a series of diseased plants, and the ejected droplets of honeydew were picked up in needles and injected into nonviruliferous nymphs. The honeydew was excreted and assayed over a period of time varying from 15 minutes to approximately 7 hours. Thirty-six such droplets were tested of which 11 proved to be infectious. The minimum access period after which an infectious honeydew droplet was excreted was approximately 1.5 hours.

An experiment was done to determine how long aphids that have left a diseased plant would continue to excrete infectious honeydew. Thirty nonviruliferous aphids were allowed access to virus source plants for 12 hours at 20°C. The aphids were then transferred to 30 healthy sweetpea seedlings, one aphid per plant, and every 24 hours the insects were moved to new test plants for a total of 7 days. The first honeydew droplets excreted by each aphid upon leaving the virus source plants were assayed, and 19 aphids were found to have excreted infectious honeydew. But when the honeydew excreted by the donor aphids on the 2nd, 3rd, and the 7th day of feeding on healthy plants was assayed, only 2, 1, and 0 aphids were found to have excreted infectious honeydew on these days, respectively. Twenty-three of the 30 aphids acquired virus during the 12-hour acquisition access period, but only 18 of these excreted infectious honeydew. In one case a honeydew droplet was infectious although the aphid from which it came failed to transmit virus to any of the 7 test plants upon which it subsequently fed.

The honeydew excreted by pea aphid apterae feeding on sweetpea plants is slightly alkaline, as tested by pH paper. This is close to the value of 7.8–8.0 given by Auclair (*1*) for pea aphids feeding on pea, *Pisum sativum* L. Limited dilution tests have indicated that honeydew can be diluted to 10^{-1} with either distilled water or 0.1 *M* phosphate buffer, pH 7.1, and still remain infectious to inoculated aphids.

Of all the sources of inoculum we have tested, *viz.*, plant extracts, hemolymph from viruliferous aphids, and honeydew excreted by aphids feeding on virus-infected plants, the last-mentioned appears to be the most promising. Honeydew of the pea aphid is readily collected, and a single droplet from an adult is sufficient to inoculate several insects.

Honeydew is qualitatively similar in composition to the phloem sap of the plant on which the aphids feed (*8*) and from which the pea-enation mosaic virus is presumably acquired. Honeydew contains a mixture of excess carbohydrates, amino acids, water, and other phloem products, with some additional metabolic and excretory materials from the aphids (*19*), but apparently lacks any significant amount of protein (*1*). The honeydew excreted by the pea aphid is clear

and, in spite of the obvious quantitative limitations, should serve as a good medium from which to concentrate or purify virus. Perhaps virus particles could be visualized by electron microscopic examination of honeydew.

Preliminary work has also indicated the suitability of the injection technique using virus-bearing honeydew for the determination of pH stability and dilution studies. It should prove equally adaptable for determining other properties of viruses.

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The Development of Ultraviolet-Irradiation Resistance by Poliovirus Infective Centers and Its Inhibition by Guanidine[1]

Guanidine, a selective inhibitor of picornavirus reproduction, has been shown to inhibit the synthesis of viral RNA polymerase and of viral RNA [cf. review by Tamm and Eggers (1)]. The problem as to when in the multiplication cycle of poliovirus type 1 the synthesis of virus-directed macromolecules begins has been investigated in kinetic experiments in which guanidine was removed by washing the cultures at different times after infection. The results indicated that the guanidine-inhibitable period begins about 1 hour after virus inoculation (*2*), but biologic and isotope data showed that guanidine is not readily removed from cell cultures by washing with guanidine-free medium (*2*). The possibility could therefore not be excluded that the guanidine-sensitive process actually begins somewhat later than inferred from the kinetic experiments.

A different approach to the problem of the early events in poliovirus replication is available in the Luria-Latarjet type of experiment (*3*) as applied to the poliovirus system (*4*). In the present study, the acquisition of ultraviolet (UV)-irradiation resistance by infective centers was followed in the presence or absence of guanidine. For comparison, infected cells treated with puromycin were also examined. The removal of the compound from the cells is not a necessary part of this experimental design.

The results of a typical experiment with guanidine-sensitive poliovirus 1 (Brunenders) are presented in Fig. 1. In addition, the inactivation kinetics of the free virus are shown. UV-irradiation of infective centers was performed after a total incubation period at 37° of 50, 110, and 180 minutes following virus inoculation. The slope of the inactivation curve of the infective centers from untreated cultures, determined at 50 minutes following infection, is slightly less steep than that of free virus, in agreement with results obtained by other investigators (*8, 9*). Not illustrated in the graph is the

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